MEIOTIC *TRANS*-SENSING AND MEIOTIC SILENCING IN *Neurospora crassa*

A Dissertation

by

ROBERT J. PRATT II

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Microbiology

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Approved by:

Chair of Committee,	Rodolfo Aramayo
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ABSTRACT

Meiotic *Trans*-sensing and Meiotic Silencing in *Neurospora crassa*.

(December 2008)

Robert J. Pratt II, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Rodolfo Aramayo

Meiosis, the core engine of sexual reproduction, is a complex process that results in the production of recombinant haploid genomes. In the meioses of Neurospora, worms and mice, gene expression from DNA that lacks a pairing partner is silenced. We posit that this is a two-step process. First, a process called meiotic *trans*-sensing compares the chromosomes from each parent and identifies significant differences as unpaired DNA. Second, if unpaired DNA is identified, a process called meiotic silencing inhibits expression of genes within the unpaired region and regions sharing sequence identity. Meiotic silencing is mechanistically most likely related to RNAi in other eukaryotes.

We used a combination of forward and reverse genetic strategies aimed at understanding the mechanisms of meiotic *trans*-sensing and meiotic silencing. Here, we present genetic evidence that arguably differentiates the meiotic *trans*-sensing step from meiotic silencing, by demonstrating that DNA methylation affects sensing of specific allele-types without interfering with silencing in general. We also determined that DNA sequence is an important parameter scrutinized during meiotic *trans*-sensing. This, and other observations, led us to hypothesize meiotic recombination as the mechanism for meiotic *trans*-sensing. However, we find that mutants of key genes required for recombination and chromosome pairing are not required for locus-specific meiotic silencing. We conclude that two interesting possibilities remain: meiotic *trans*-sensing occurs

through a previously uncharacterized recombination pathway or chromosomal regions are carefully compared in the absence of recombination. Finally, forward genetics revealed a novel component of meiotic silencing, *Sms-4*, encoding the Neurospora ortholog of mammalian mRNP component ELG protein. Unlike previous loss-of-function mutants that abate meiotic silencing by unpaired DNA, *Sms-4* is not required for successful meiosis, showing that meiosis and meiotic silencing are distinct, yet overlapping, phenomena. Intriguingly, SMS-4 is the first component to be localized with bulk chromatin in the nucleus, presumably the site of *trans*-sensing. Finally, we carried out a critical examination of the current evidence in the field and present alternative models for meiotic *trans*-sensing and meiotic silencing in Neurospora.

DEDICATION

This dissertation is dedicated to my family and my wife, Lida Silvana Paredes Martinez, for all of their support and love.

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CHAPTER I

INTRODUCTION

MEIOTIC SILENCING BY UNPAIRED DNA

In the meioses of Neurospora, worms and mice, gene expression from DNA that lacks a pairing partner is silenced. This meiotic silencing by unpaired DNA was first observed in *Neurospora crassa* when it was shown that expression of a gene required for the pigmentation of sexual spores required meiotic pairing for proper regulation (Aramayo and Metzenberg 1996). This was reminiscent of transvection previously only observed in Drosophila (Lewis 1954). Further investigations suggested that, rather than gene expression being activated by pairing, gene expression may be prevented by unpairing, probably by triggering RNAi-like silencing of homologous mRNAs (Lee *et al.* 2003b, Shiu *et al.* 2001).

Since then, meiotic silencing by unpaired DNA has been discovered in both *C. elegans* and mouse. Here, unpaired DNA is associated with specific histone modifications and transcriptional silencing (Baarends *et al.* 2005; Bean *et al.* 2004; Turner *et al.* 2005). In worms, unpaired DNA is imprinted and re-gains transcriptional activation at a slower rate than paired regions due to its ability to retain chromatin signatures related to silencing longer in the zygote (Bean *et al.* 2004). In mouse, meiotic silencing might be related to the meiotic sex chromosome inactivation (MSCI) observed during male spermatogenesis (Handel 2004). The role of this inactivation is controversial, but has been proposed as a mechanism for imprinting the paternal X-chromosome for preferential inactivation in the murine placental tissues and marsupial embryos (Huynh and Lee 2005; Okamoto *et al.* 2005). In both mouse and worms, there is a strong correlation between homologous and non-homologous synapsis and transcription (Bean *et al.* 2004; Turner *et al.* 2006; Turner *et al.* 2005). In addition to the silencing of these large chromosomal regions, smaller unpaired regions in mouse spermatogenesis also seem to invite heritable epigenetic

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This dissertation follows the style of Genetics.

modification and may be mechanistically related to meiotic silencing in Neurospora (Herman *et al.* 2003; Rassoulzadegan *et al.* 2006; Rassoulzadegan *et al.* 2002).

While there are differences in the known properties of these phenomena, there is one key underlying theme: They all seem to identify regions of unpaired DNA and target them for silencing. These silencing phenomena are considered epigenetic, because they results in a heritable, yet reversible, mutant phenotype in progeny that contain genes with a wild-type DNA sequence. By studying meiotic silencing in the genetically malleable and fast growing filamentous fungus *Neurospora crassa*, we hope to shed light on the properties and mechanisms of these and other potentially related epigenetic phenomena.

RNA SILENCING

In Neurospora, meiotic silencing appears to be an RNAi-like mechanism. RNAi is an evolutionarily conserved pathway whose core components were probably present in the last common ancestor of all eukaryotes (Cerutti and Casas-Mollano 2006), in which it may have served a role in silencing parasitic nucleic acids, as it does still in many eukaryotes (Aravin *et al.* 2001; Djikeng *et al.* 2001; Kalmykova *et al.* 2005; Kuhlmann *et al.* 2005; Nolan *et al.* 2005; Sijen and Plasterk 2003; Tabara *et al.* 1999; Wu-Scharf *et al.* 2000; Zilberman *et al.* 2003). At least three enzymes seem to make up the core of the RNAi pathway, based on their requirement for RNAi in many organisms across eukaryotic lineages: the Argonaute, the RNA-dependent RNA polymerase (RdRP) and the Dicer (Cerutti and Casas-Mollano 2006).

The generalized model for RNAi initiates with the creation of dsRNA. This can be introduced exogenously, encoded endogenously, or result from the conversion of a ssRNA to dsRNA via RdRP. DsRNA is then diced into smaller

20-25 nucleotide small interfering RNAs (siRNAs) by Dicer. These siRNAs would then enter a complex containing an Argonaute, which would use the sequence of the siRNA to identify target molecules based on sequence complementarily (Allis *et al.* 2006; Hammond 2005; Meister and Tuschl 2004). Meiotic silencing in Neurospora requires the activities of each of these core components, namely *Sad-1*, an RdRP, *Sms-3*, a Dicer, and *Sms-2*, an Argonaute (Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu *et al.* 2001).

Outside this core, other components required for RNAi in different organisms have either changed significantly at the sequence level or may have been added to the pathway later to diversify the functions of RNAi. The ancestral RNAi pathway was likely capable of directing post-transcriptional silencing and transcriptional silencing of transposable elements (Cerutti and Casas-Mollano 2006). However, these machineries have been recruited into other biological roles, including development of centromeric chromatin (Volpe *et al.* 2003), programmed DNA elimination (Mochizuki *et al.* 2002), translational repression of mRNAs during development (Ambros 2003), and the silencing of unpaired DNA in meiosis (Lee *et al.* 2003b; Shiu *et al.* 2001).

NEUROSPORA BIOLOGY

To understand these investigations into meiotic silencing in *N. crassa*, a basic understanding of *N. crassa* biology is needed (reviewed Davis 2000) (Figure 1). *N. crassa* is a heterothallic filamentous fungus with two non-switching mating-types, A and a. It grows vegetatively as a multinucleate mass of interconnected hyphal cells called mycelia. Asexual reproductive development involves the production of aerial hyphae that form chains of terminal asexual spores called conidia. Conidia can germinate to form a new mycelia network, thereby completing the vegetative cycle. Under poor nitrogen conditions, sexual development of a female structure called a protoperithecium occurs. Special

receptor hyphae, called trichogynes, emanate from the protoperithecium. These will fuse with a male element of the opposite mating-type during fertilization. Following fertilization, the fruiting body hardens and darkens and is referred to as a perithecium. Neurospora is also hermaphroditic, so strains of either mating-type can be the female or the male. Inside the perithecium, surrounded by maternal tissue (paraphysal hyphae), nuclei of both mating partners divide in the heterokaryotic ascogenous hyphae. Prior to meiosis, nuclei of opposite mating-type undergo a synchronous mitosis to isolate two nuclei in the tip of a specialized structure called the crozier. Here the nuclei fuse to form a diploid nucleus during karyogamy. The diploid nucleus undergoes meiosis followed by a round of mitosis to yield eight sexual spores called ascospores within an ascus in an order that reflects their lineage. Ascospores are then forcefully ejected from a hole in the perithecium. Following brief exposure to high temperature (~60ºC for 40 minutes), ascospores can germinate to produce vegetative mycelia. Within a perithecium, multiple asynchronous meioses occur simultaneously. These developing tissues can be dissected from the perithecium and connected as a rosette of asci.

GENOME DEFENSE IN NEUROSPORA

Neurospora seems to be pathologically paranoid of invasion by nucleic acids. Invaders could find at least four levels of resistance to their propagation in this host. If the invader is relatively AT-rich or becomes repetitive through duplication events it could be transcriptionally silenced and methylated at the DNA level (Freitag *et al.* 2001; Romano and Macino 1992; Singer *et al.* 1995; Windhofer *et al.* 2000; Zhou *et al.* 2001). Additionally, if the invader DNA is repetitive or has the misfortune of inserting in a way such that its transcript creates dsRNA, it could be silenced during vegetative growth by an RNAi-like mechanism called quelling (Cogoni *et al.* 1996; Nolan *et al.* 2005; Pickford *et al.* 2002; Romano and Macino 1992).

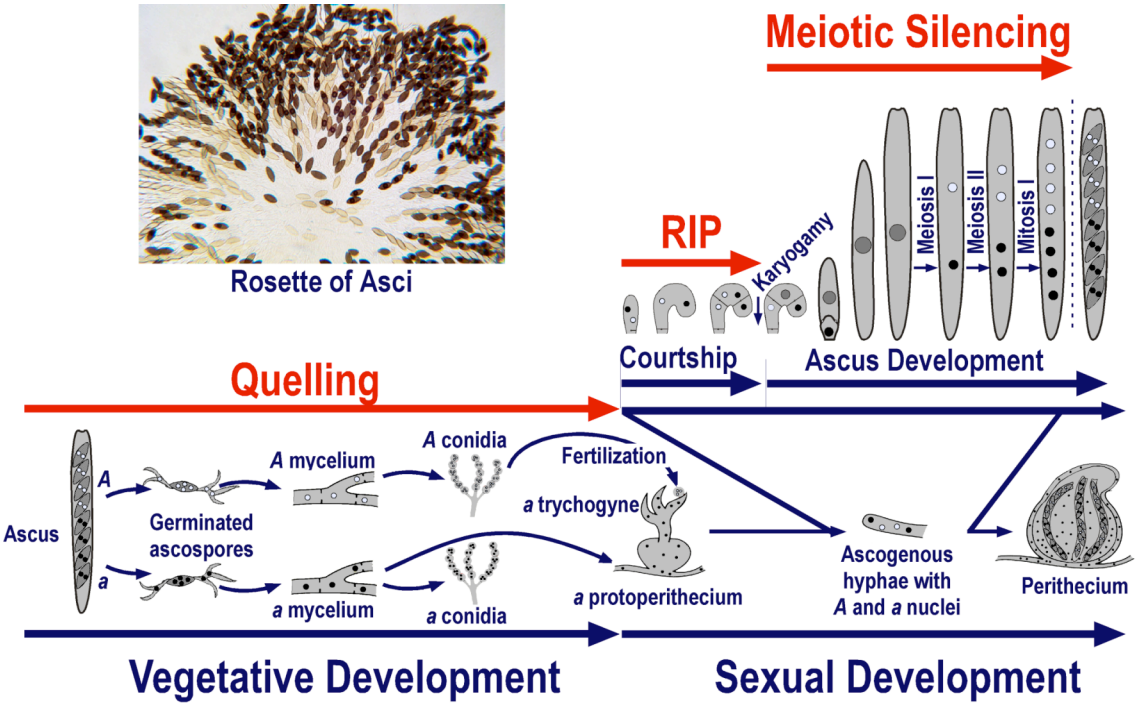

Figure 1. The life cycle of *Neurospora crassa*. Stages of development are designated by blue arrows. The developmental windows during which the genome defense mechanisms of quelling, RIP and meiotic silencing are proposed to work are designated by red arrows. Modified from Borkovich *et al*. (2004). See text for details.

Even if it manages to replicate itself only once, and is of sufficient size (>500-bp), it will experience trouble if the host enters the sexual cycle. Prior to meiosis, each host genome is scanned for such duplications, and if present, these can be savagely inactivated by repeat induced point-mutation (RIP). This process introduces numerous GC to AT transition mutations, methylates the DNA, and silences the regions by generating repressive chromatin (Kinsey *et al.* 1994; Rountree and Selker 1997; Selker 1990; Selker *et al.* 1987; Selker *et al.* 2002; Tamaru and Selker 2001). As if this were not enough, when the host genome enters meiosis, it is compared with the genome of its mating partner through a process called meiotic *trans*-sensing (Aramayo and Metzenberg 1996; Lee *et al.* 2004; Pratt *et al.* 2004). Any extra DNA detected, e.g., a novel transposon insertion, gets silenced through the course of meiosis by a second RNAi-like mechanism called meiotic silencing by unpaired DNA (sometime sharing the

acronym MSUD with maple syrup urine disease) (Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu *et al.* 2001).

With these silencing mechanisms, Neurospora can arguably protect its genome from parasitic nucleic acids during all stages of its life cycle (Figure 1). Providing evidence for the effectiveness of these strategies, there is only one known functional transposable element in Neurospora, and this is present in only one of more than 300 Neurospora isolates from around the world (Kinsey 1989). Additionally, when this transposable element was introduced into laboratory strains, its copy number was repressed in a quelling-dependent manner (Nolan *et al.* 2005) and duplications were inactivated by RIP (Kinsey *et al.* 1994). Testimony to other transposable elements that have tried their luck in Neurospora and failed is the presence of their RIP-mutagenized relics remaining in the genome (Selker *et al.* 2003).

MEIOTIC *TRANS*-SENSING AND MEIOTIC SILENCING

Unlike quelling and RIP, there is no direct evidence that meiotic silencing is effective in preventing the spread of transposable elements in meiosis. However, it seems that it could be effective. To illustrate this idea in the context of Neurospora meiosis, we present Figure 2. The foundations for this model will be discussed in detail in Chapter V.

Let's imagine sex between two haploid parents (parents A and a) where one of them obtained a transposable element (green) in the previous vegetative cycle. We will focus on only one homologue pair. These chromosomes undergo pre-meiotic DNA replication, creating sister chromatids attached along their length by cohesin. The nuclei will fuse during karyogamy, creating a diploid nucleus. During prophase I of meiosis, these homologous chromosomes are going to search for each other among the other non-homologous chromosomes in the

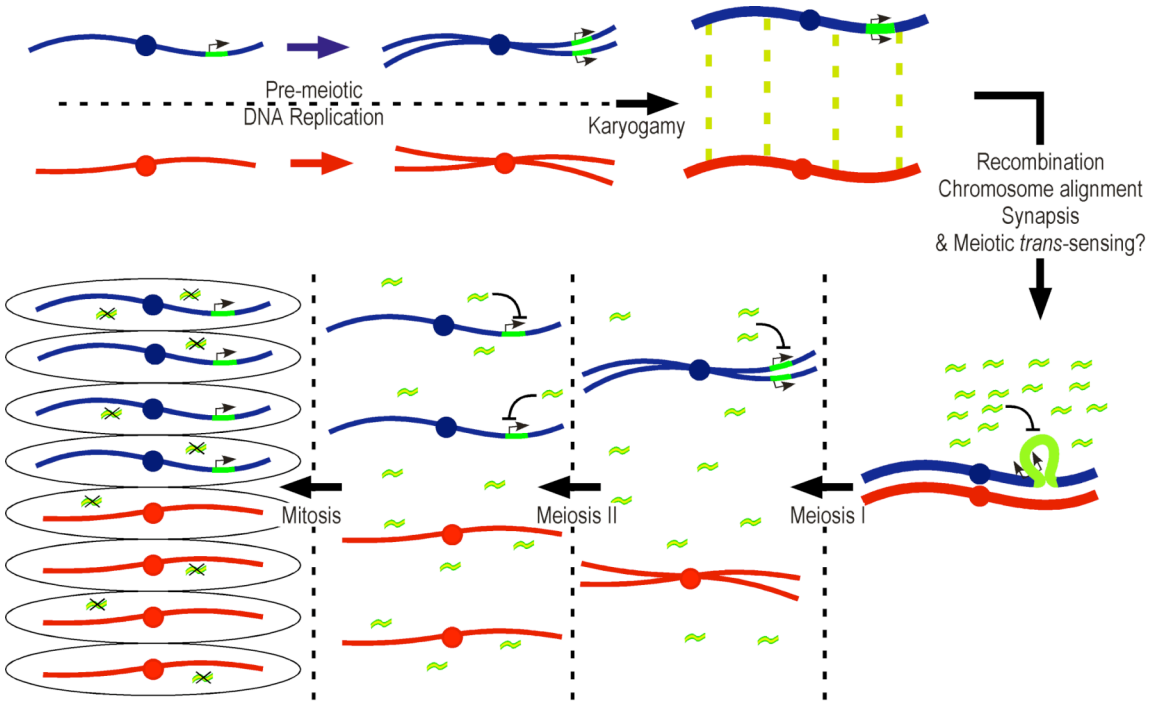

Figure 2. Meiosis, meiotic *trans*-sensing and meiotic silencing. Red and blue lines represent homologous chromosomes from two mating parents, Parent *A* and Parent *a*. The black horizontal dashed line delineates haploid pre-karyogamic parents, and the vertical black dashed lines delineate nuclear divisions. Yellow dashed lines represent the active comparison between chromosomal regions that occurs during recombination, and perhaps during meiotic *trans*-sensing. Sequential events are indicated by black arrows. The green region on one chromosome is a transposable element. The small green squiggly lines represent a diffusible signal containing the sequence information from the unpaired DNA. This sequence information is used by meiotic silencing to silence homologous regions during meiosis (barred lines). See text for details.

nucleus and pair. They will initiate recombination by introducing programmed double-stranded breaks in their chromosomes, align at a distance, then become progressively closer as a proteinaceous structure called the synaptonemal complex forms between the homologues (reviewed Raju 1980; Zickler 2006).

It is also during prophase I of meiosis that meiotic *trans*-sensing likely occurs, which we define as the mechanism by which homologous chromosomes are compared and unpaired DNA is identified. For instance, in our model, the new transposable element, along with all of the other regions on the homologous chromosome, will undergo *trans*-sensing with the regions on the homologue.

However, the transposable element will fail to detect a homologous region at the allelic location on its homologue and will be identified as unpaired DNA.

In Neurospora this would have a meaningful consequence, because the detection of this unpaired DNA would trigger meiotic silencing. This would result in the production of a signal, perhaps siRNAs, that would target the loss of transcripts from the unpaired region and any other region of the genome with sequence identity. Meiotic silencing continues as homologous chromosomes separate at the end of meiosis I, as sister chromatids separate at meiosis II and as a post-meiotic mitosis creates eight haploid nuclei. The nuclei are then encapsulated into individual ascospores and meiotic silencing stops.

ASSAYING MEIOTIC SILENCING

Experimentally, meiotic silencing is assayed by performing crosses in which a reporter gene, typically a native gene required for ascospore morphology or color, is unpaired. In this way, the degree of meiotic silencing can be measured as a function of the relative levels of ascospores with mutant and wild-type phenotypes. An example with the reporter gene *Round spore* (*Rsp*) and the meiotic silencing component *Sad-1* is shown in Figure 3. In a cross between a wild-type strain and a strain containing loss-of-function allele of *Rsp*, e.g., a single-nucleotide insertion creating a frame-shift (*rsp*^{fs}), the *Rsp* alleles from both parents detect sufficiently homologous regions on their homologous chromosomes by *trans*-sensing. As a result the single *rsp*$^{+}$ allele provides sufficient functional product and all of the ascospores have a spindle-shaped morphology (Figure 3A). However, crosses homozygous for a loss of function in *Rsp* produced asci containing eight round ascospores.

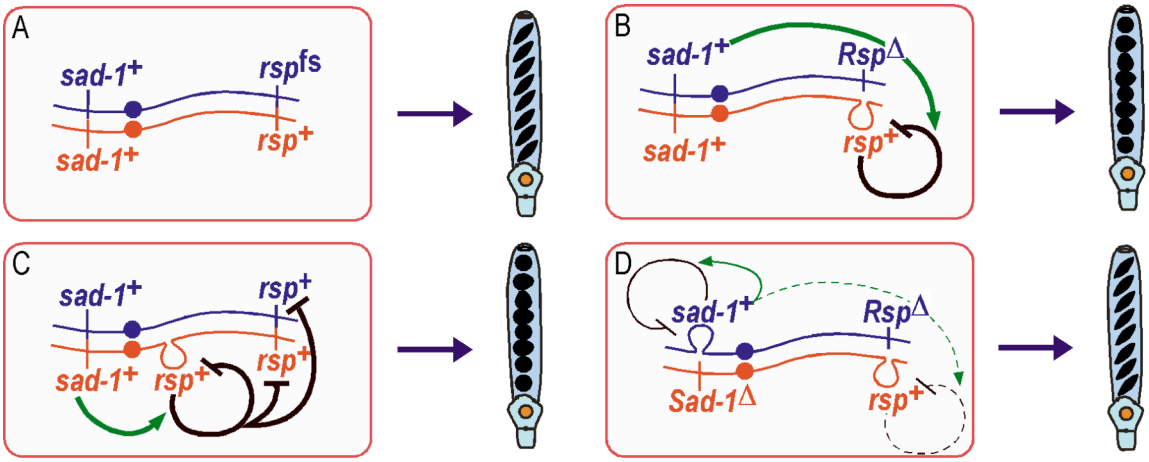

Figure 3. Assaying meiotic silencing with *Rsp*. A diploid meiosis I nucleus is depicted as a red box. One homologous chromosome pair (red and blue) is depicted with the *Rsp* and *Sad-1* loci specified. An ascus with eight ascospores of the resulting phenotype is shown to the right of the nucleus. Green arrows represent *Sad-1* activity and black bars represent silencing. Thickness of these lines represents relative levels of silencing or activity. A) Paired *rsp*$^+$. B) Unpaired *rsp*$^+$ resulting in *cis*-silencing. C) An ectopic *rsp*$^+$ resulting in *trans*-silencing. D) Suppression of meiotic silencing of unpaired *rsp*$^+$ by the unpairing of *sad-1* $^+$.

In contrast to the situation with a small frame-shift mutation in *Rsp*, in crosses to a deletion of *Rsp* (*Rsp*Δ) the *rsp*$^+$ allele fails to sense its homologue and is silenced through the activities of sad-1 and the other gene products required for meiotic silencing. As a result, in the asci where silencing is effective, all ascospores have a round or ovoid morphology (Figure 3B). Therefore the *Rsp*Δ allele is ascus-dominant and violates Mendelian law (at least at the level of phenotype) since all ascospores appear mutant, even those that contain functional *rsp*$^+$. Similarly, some RIP alleles (mutant alleles generated through RIP) behave dominant in meiosis despite the fact that these RIP alleles are of identical length and can be 94% identical at the DNA level to the wild-type allele (Pratt *et al.* 2004). We refer to these previous two classes of meiotic silencing inducers as indels and homeologous regions, respectively. Both of these induce *cis*-silencing, where only the DNA that is unpaired needs to be silenced.

Alternatively if a wild-type strain is crossed to a partner containing an ectopic copy of *Rsp* (*e.g.,* by directed insertion at the *his-3* locus), the ectopic allele will be unpaired and will trigger the silencing of all *Rsp* alleles regardless of whether the other alleles are themselves paired or unpaired. Again round ascospores are produced (Figure 3C). We refer to this type of silencing as *trans*-silencing, where paired regions must be silenced.

Another way in which meiotic silencing is assayed is by unpairing a gene required for meiotic silencing and assay the level of silencing of a second unpaired reporter. For example if one parent contains a deletion of *Rsp* and the other contains a deletion of *Sad-1*, both genes are unpaired. For reasons that are not yet clear, *Sad-1* appears to silence itself, reducing its function to a level that prevents it from efficiently silencing unpaired *Rsp* (Figure 3D). We call this suppression of meiotic silencing, and this dominant suppression behavior is shared by all known components of meiotic silencing, perhaps partly due to the selection strategies used to identify them. It should be noted, however, that not all loss of function alleles of meiotic silencing components are dominant and that the dominance of the alleles follows the same rules for pairing discussed in Chapter V for other reporter genes.

Ultimately, the output of these experiments is, by convention, a percentage of wild-type ascospores or asci. The stronger the silencing of the reporter gene, the lower the percentage of ascospores or asci with a wild-type phenotype. Alternatively, the stronger the suppression of meiotic silencing, the higher the percentage. But what does this number really mean? It is the output of two events, meiotic *trans*-sensing and meiotic silencing, and the relative contribution of each to this percentage is still unclear. For example, one can imagine that once induced, meiotic silencing is always 100% effective within an ascus. If so, the output is a direct measure of the efficiency of meiotic *trans*-sensing, or of the

frequency at which unpaired DNA is detected. The converse is also potentially true; unpaired DNA is always detected but the efficiency of meiotic silencing varies. Additionally, of course, the output could be a combination of both efficiencies.

RESEARCH AIMS

The long-term objectives are to determine the mechanisms by which homologous regions are compared in meiosis and in general, i.e., *trans*-sensing, and to determine how unpaired DNA is silenced. The specific objectives of the research presented here are: 1) to investigate the aspects of RIP alleles that influence the susceptibility of their pairing partners to meiotic silencing; 2) to investigate the role of critical recombination and synapsis components in meiotic *trans*-sensing and meiotic silencing; and 3) to characterize a new gene required for meiotic silencing, *Sms-4*.

CHAPTER II

DNA METHYLATION AFFECTS MEIOTIC *TRANS*-SENSING, NOT MEIOTIC SILENCING*

INTRODUCTION

Homology-sensing mechanisms are at center stage in biology (Wu and Morris 1999). Complex genomes have evolved sophisticated ways to sense the presence and to control the behavior of repeated DNA sequences. At risk is their chromosomal integrity, and with it, the very existence of the organism. The situation is more critical in meiosis, a developmental stage that requires cells to activate a series of sophisticated molecular mechanisms that will ensure precise chromosome duplication, repair and recombination (Kleckner 1996; Roeder and Bailis 2000; Villeneuve and Hillers 2001; Zickler and Kleckner 1998; Zickler and Kleckner 1999). Here, at least two things are critical. First, chromosomal integrity must be maintained. Even a small increase in the frequency of ectopic recombination between dispersed repeats would have catastrophic consequences for the genome. Second, the genetic information of homologous chromosomes must be compared to determine whether the chromosomes participating in meiosis belong to the same species.

The destructive potential of dispersed repeats has been thoroughly studied (Montgomery *et al.* 1991; Rouyer *et al.* 1987; Small *et al.* 1997). As have been the factors responsible for limiting recombination between repeats (Jinks-Robertson *et al.* 1993; Maloisel and Rossignol 1998; Radman and Wagner 1993; Rayssiguier *et al.* 1989; Shen and Huang 1986). As a result of these studies, it is increasingly clear DNA methylation plays a major role among the many molecular mechanisms involved in genome stability. For example, DNA methylation is often associated with repeat-rich intergenic regions in plants, and

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*Reprinted with permission from DNA methylation affects meiotic *trans*-sensing, not meiotic silencing, in Neurospora by R. J. Pratt, D. W. Lee and R. Aramayo, 2004. Genetics **168:** 1925-1935. Copyright 2004 by The Genetics Society of America.

is present in DNA repeats in mammals (Bennetzen *et al.* 1994; Yoder *et al.* 1997). In addition, mutation of a DNA methyltransferase and an Argonaute in mouse results in loss of methylation, transposon activation and meiotic failure (Bourc'his and Bestor 2004; Carmell *et al.* 2007).

It is therefore not surprising to find that the same molecular mechanisms used by cells to maintain their genome stability have been recruited to counteract the invasion of a genome by viruses, retrotransposons, and insertion sequences which, if unchecked, can have deadly consequences to the organism. Arguably, filamentous fungal genomes are at a greater risk than those of plants and animals because a single cytoplasm is shared by many nuclei in these organisms. Genomes like that of *Neurospora crassa* have developed a number of complex molecular mechanisms to preserve their integrity (Borkovich *et al.* 2004; Galagan *et al.* 2003). At least four distinct but potentially interrelated mechanisms are known; DNA methylation, quelling, repeat induced point mutation (RIP), and meiotic silencing (see Chapter I).

Before mating partners compare each other's genome through meiotic *trans*-sensing, each undergoes an introversive search for gene-sized duplications in their own genome. If such duplicated sequences are detected they are then subjected to a haploid-specific silencing mechanism called RIP (Freitag *et al.* 2002; Selker 1990; Selker 1997). In this process, a series of GC-to-AT transition mutations are introduced into the duplicated sequences. Many of the remaining non-mutated cytosine bases are methylated by DIM-2, a DNA methyltransferase responsible for all the known cytosine methylation in Neurospora (Kouzminova and Selker 2001). Additionally these sequences are frequently blocked in transcription elongation and associated with trimethylated histone-H3, lysine-9 (H3-K9) chromatin (or heterochromatin) (Rountree and Selker 1997; Tamaru *et al.* 2003).

This study was prompted by the need to genetically determine the requirements of meiotic *trans*-sensing. Some RIP alleles of *Sad-1* are dominant in meiosis presumably due to meiotic silencing (Shiu and Metzenberg 2002). The known changes to a gene introduced by RIP occur at the levels of DNA sequence, DNA methylation, chromatin and transcription. We hypothesized that many of these changes could together or independently contribute to the dominance of RIP alleles by affecting successful *trans*-sensing between the alleles and their wild-type homolog. Since these changes can vary widely among RIP alleles and are varyingly dependent upon the DNA methyltransferase *dim-2*, to test this hypothesis, we isolated RIP alleles of *Rsp* exhibiting a wide range of meiotic silencing and assayed the affect of DNA methylation on meiotic silencing. Importantly, if as suggested previously (Shiu and Metzenberg 2002), meiotic silencing is independent of DNA methylation, then changes in level of meiotic silencing would arguably represent changes in the efficiency of meiotic *trans*-sensing.

We observed that DNA methylation does indeed affects the dominance of some RIP alleles suggesting it affects meiotic *trans*-sensing, but not meiotic silencing in general. We further determined that in the absence of DNA methylation, the meiotic silencing triggered by the alleles tested moderately correlated with DNA identity, suggesting that DNA sequence was also an important parameter during meiotic *trans*-sensing. Together, these experiments assign a previously undescribed role for DNA methylation in meiosis, and based on studies in other systems, we speculate on the existence of an intimate connection between meiotic *trans*-sensing, silencing and recombination.

RESULTS

The DNA methyltransferase gene (*dim-2*$^{+}$) may affect meiotic *trans*-sensing, but does not affect meiotic silencing

We hypothesized that *trans*-sensing is possibly sensitive to multiple allelic differences introduced by RIP. If this were the case, among a series of lightly-RIP alleles, we should obtain *Rsp*^{RIP} alleles that would be dominant in the presence, but recessive in the absence, of DNA methylation. This prediction was confirmed. We started by isolating two *Rsp*^{RIP} alleles that we called *Rsp*^{RIP93} and *rsp*^{RIP94} (Figure 4A). DNA sequence analysis of the RIP regions revealed that these alleles are 94% and 97% identical to the wild-type region, respectively (Figure 4A).

Surprisingly, when crossed to wild-type, *Rsp*^{RIP93} was dominant and *rsp*^{RIP94} was recessive (Figure 4B), indicating that only a 6% divergence between *Rsp*^{RIP93} and *rsp*$^{+}$ and only a ~3% divergence between *Rsp*^{RIP93} and *rsp*^{RIP94} might determine their dominant behavior in meiosis. To test for the effects of DNA methylation on these alleles, we constructed double mutants between each one of these RIP alleles and *dim-2*. For this we used *dim-2*(1), an allele containing a nonsense mutation (Kouzminova and Selker 2001). When crossed to *rsp*$^{+}$ *dim-2* strains, the genetic behavior of *rsp*^{RIP94} allele remained unchanged. In contrast, we observed a dramatic change for *Rsp*^{RIP93}, from dominant to semi-recessive (Figure 4B).

It was formally possible that the removal of DNA methylation was relieving the transcriptional repression of a highly mutant but ultimately functional *Rsp*^{RIP93} allele. This possibility was formally discarded based on two observations: First, mutations introduced by RIP obliterated the translational start signal of the *Rsp*^{RIP93} DNA region and introduced a series of in frame stop codons (data not

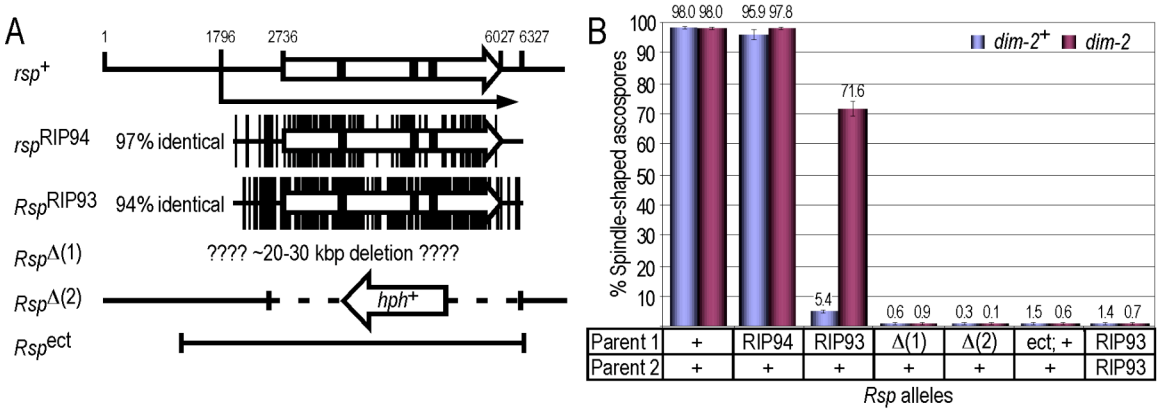

Figure 4. The DNA methyltransferase gene (*dim-2*$^+$) can affect meiotic *trans*-sensing, not meiotic silencing. (A) The *Rsp* region. On top, a diagram of the *Rsp* locus indicating the positions of the transcription start site (coordinate 1796), predicted translation start and stop sites (coordinates 2736 and 6027, respectively), and polyadenylation site (coordinate 6327) are presented, along with the relative positions of the five exons (white rectangles) and three introns (black rectangles) in the region. The arrow spans the region corresponding to the *Rsp* transcript. Below, diagrams of the molecular structures of the different *Rsp* alleles are presented. The alleles *rsp*^{RIP94} and *Rsp*^{RIP93} were both constructed by RIP of the *rsp*$^+$ allele by duplicating the 4,391 bp region (coordinates 1962 to 6353) at the *histidine-3* (*his-3*) locus. The percent of identity indicated for the region is relative to the *rsp*$^+$ wild-type allele. Each vertical bar represents one point mutation. Alleles *rsp*^{RIP94} and *Rsp*^{RIP93} have 129 and 254 mutations present along the duplicated region, respectively (Table 1). Allele *Rsp*$^{\Delta(1)}$ is a large natural deletion estimated to be between 20 and 30 kbp in length. Allele *Rsp*$^{\Delta(2)}$ was constructed by replacing a 3.8 kbp region corresponding to the coding, leader and minimal promoter regions of *rsp*$^+$ with the hygromycin B phosphotransferase (*hph*$^+$) gene selection marker. Strains carrying *Rsp*^{ect} allele, contain the 5,252 bp fragment corresponding to the promoter and coding region of *rsp*$^+$ (coordinates 1123 to 6375) integrated at the *his-3* locus in a *rsp*$^+$ (wild-type) background. (B) *dim-2*$^+$ is an allele-specific enhancer of meiotic silencing. The column plot presents the percentage of spindle shaped ascospores observed for the different crosses in the presence (*dim-2*$^+$) or absence (*dim-2*) of DNA methylation. The higher the number of spindle-shaped ascospores, the lower the degree of meiotic silencing. Each percentage number indicated is the average of at least three crosses with a mean of 971 ascospores counted per cross. The *Rsp* genotype of each parent in the cross is indicated below.

shown). Second, *Rsp*^{RIP93} homozygous crosses produced all round ascospores, regardless of their methylation state (Figure 4B). Together these observations demonstrate the non-functional nature of the *Rsp*^{RIP93} allele.

To demonstrate that DNA methylation was only affecting *trans*-sensing but not meiotic silencing, we compared the meiotic silencing of deletions or insertion alleles of *Rsp* when crossed to wild-type, either in the presence or absence of DNA methylation. As expected, crosses between *Rsp*$^{\Delta}$ alleles and *rsp*$^{+}$, (*i.e.,* both [*Rsp*$^{\Delta(1)}$ x *rsp*$^{+}$] and [*Rsp*$^{\Delta(2)}$ x *rsp*$^{+}$], Figure 4B) and between a *Rsp*-duplication and *rsp*$^{+}$ (*i.e.,* [*rsp*$^{+}$*, Rsp*^{ect} x *rsp*$^{+}$], Figure 4B), produced abundant progeny with round spores in both the presence or absence of DNA methylation. These results are consistent with previous observations (Shiu and Metzenberg 2002), and strongly support the notion that DNA methylation does not affect meiotic silencing in general.

Although unlikely, it was also formally possible that the suppression observed for the *Rsp*^{RIP93} allele was due to the presence of a mutation linked to the original *dim-2*$^{(1)}$ mutant allele (Kouzminova and Selker 2001). To test for this possibility, we generated two new mutant alleles of *dim-2* by RIP (called *dim-2*^{RIP89} and *dim-2*^{RIP90}). When combined with the *Rsp*^{RIP93} allele and tested, similar levels of suppression were obtained (Figure 5). These experiments directly implicate *dim-2* loss-of-function alleles as allele-specific suppressors of meiotic silencing.

In summary, we conclude that the meiotic silencing induced by, and thus the dominance of, the *Rsp*^{RIP93} allele is mostly due to DNA methylation. These data are consistent with a model in which DNA methylation, directly or indirectly, interferes with meiotic *trans*-sensing but not with meiotic silencing.

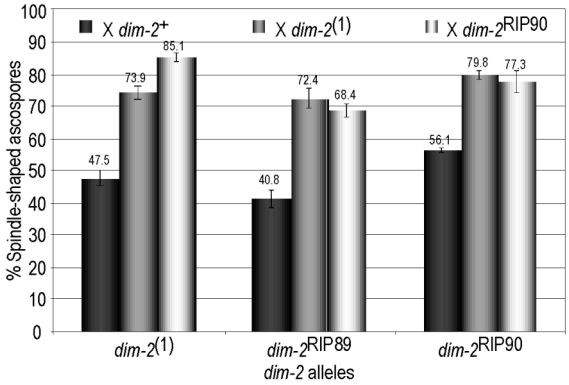

Figure 5. The dominance of *Rsp*RIP93 is also suppressed by *dim-2*RIP alleles. Strains double mutants for *Rsp*RIP93 and *dim-2* (x-axis), were crossed to strains containing either *dim-2*(1) (darkest bar), *dim-2*RIP89 (dark bar), and *dim-2*RIP90 (light bar). Each bar represents an average of at least four crosses and a mean of 987 ascospores counted per cross.

In the absence of DNA methylation, different *Rsp*RIP alleles induce different degrees of meiotic silencing

The loss of meiotic silencing associated with *Rsp*RIP93 was not absolute (*i.e.,* 71.6% as opposed to >95% spindle-shaped ascospores observed for *rsp*RIP94 crosses, Figure 4B), which suggested that parameters other than DNA methylation itself are important, and perhaps actively used during meiotic *trans*-sensing. We tested if we could isolate *Rsp*RIP alleles that were dominant in a DNA methylation-independent manner by generating a new series of *Rsp*RIP alleles and by screening among them, for those that were dominant in *dim-2*(1) homozygous crosses (see Materials and methods). Thirty-five new *Rsp*RIP alleles were isolated. Of those, eleven (*Rsp*RIP95 to *Rsp*RIP105), representing a range of DNA methylation-independent dominance were selected for further analysis (Figure 6A).

The results of crosses between each of these alleles and wild-type (*i.e., Rsp*RIP *x rsp*+), homozygous for *dim-2*+ or for *dim-2* are shown in Figure 6A. Similar to *Rsp*RIP93, alleles *Rsp*RIP99 to *Rsp*RIP105 showed a partial DNA methylation-dependent dominance. In contrast, unlike *Rsp*RIP93, alleles *Rsp*RIP95 thru *Rsp*RIP98 were dominant in a DNA methylation-independent manner. These results suggest DNA methylation is not the only parameter determining the level of meiotic silencing induced by *Rsp*RIP alleles in meiosis.

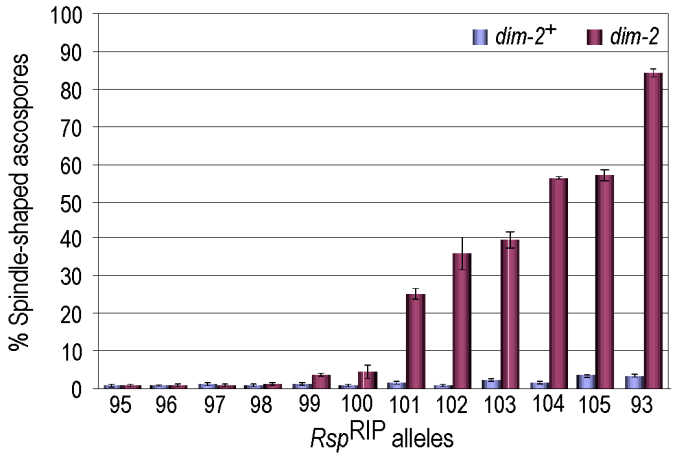

Figure 6. Characterization of new *Rsp*RIP alleles. (A) The meiotic dominance of *Rsp*RIP alleles, in the absence of DNA methylation, is variable. The presentation is as in Figure 4. All crosses were heterozygous for the different *Rsp*RIP alleles and the *rsp*+ wild-type allele and homozygous for either the *dim-2*+ (wild-type, light bar), or *dim-2* (mutant, dark bar). Each percentage number presented is the result of assaying two *dim-2*+ or four *dim-2* crosses for each allele with a mean of 1,038 ascospores counted per cross.

Ideally, the presence or absence of DNA methylation on the different *Rsp*RIP alleles would be determined in cells undergoing meiosis. This is not possible because the developing meiotic cells are immersed in maternal tissue and cannot be isolated in a pure form from Neurospora. The best experiment we can do is to determine the methylation state of the alleles in vegetative tissues, which should represent, at least, the methylation state of the nuclei entering the

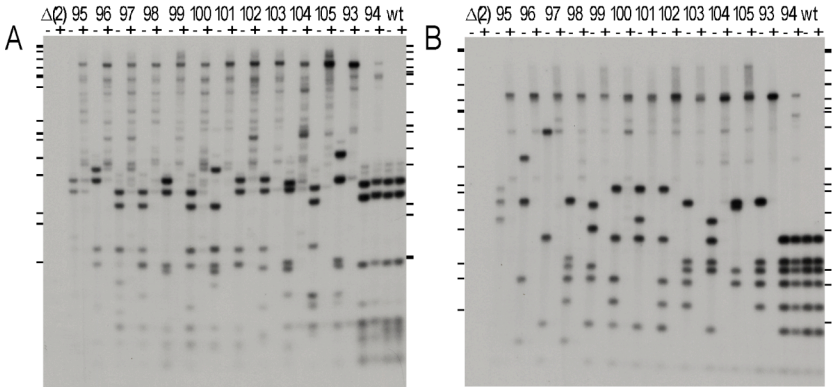

Figure 7. DNA methylation and mutation of the new *Rsp*RIP alleles. The different *Rsp*RIP alleles are methylated and demethylated in *dim-2*$^+$ and *dim-2* backgrounds, respectively. DNA from *dim-2*$^+$ (wild-type, +), or *dim-2* (mutant, -) strains each carrying either a different *Rsp*RIP allele (identified by their numbers), or the wild-type (wt) allele, was extracted and processed as described in the text. The black bars flanking the autoradiograms represent the relative positions of DNA fragments corresponding to the molecular weight markers consisting on the mixture of lambda DNA digested with *Hind*III only plus lambda DNA digested with both *Hind*III and *Eco*RI. Sizes in kbp are as follows: 22, 9.4, 6.6, 5.0, 4.4, 4.3, 3.5, 2.3, 2.0, 1.9, 1.6, 1.4, 0.95, 0.83, and 0.56. (A) *Sau*3AI digested DNA. (B) *Ava*II digested DNA.

cross. We therefore extracted DNA from vegetative tissues of strains carrying the different *Rsp*RIP alleles in a *dim-2*$^+$ and *dim-2* background. DNA was digested with the methylation-sensitive enzyme *Sau*3AI, fractionated by electrophoresis on a 1.5% agarose gel, transferred to Nylon membranes, and probed with radiolabeled fragments corresponding to the *rsp*$^+$ allele. Relative to the wild-type band patterns observed in the methylated or demethylated condition, the band-shifts observed in the demethylated condition reflect the restriction enzyme sites mutated by RIP on each allele. In contrast, for each allele, relative to the band pattern observed in the demethylated condition, the band-shifts detected in the methylated condition represent those restriction sites affected by the DNA methylation in the region. Under these conditions, with exception of the *rsp*RIP94 allele, the frequency of available sites blocked by DNA methylation was similar for all the alleles examined (Figure 7A), suggesting that these alleles are indeed methylated and demethylated in a *dim-2*$^+$ and *dim-*

2 background, respectively. Similar results were obtained with the methylation-sensitive enzyme *Ava*II (Figure 7B).

The lower the sequence identity of the different *Rsp*^{RIP} alleles to wild-type, the higher the meiotic silencing observed

In addition to DNA methylation, RIP introduces a series of transition mutations, which in turn change the DNA sequence of the region. It was therefore of interest to test if the meiotic silencing observed for the newly-isolated *Rsp*^{RIP} alleles correlated with their degree of sequence identity to the *rsp*$^{+}$ wild-type region. This was done in three steps. First, we cloned and sequenced alleles *Rsp*RIP97, *Rsp*RIP100, *Rsp*RIP102, and *Rsp*RIP103. The percent identity of these alleles when compared to wild-type is: 91.0%, 91.7%, 94.0%, and 92.8%, respectively (Table 1). Second, genomic DNA extracted from strains carrying alleles *Rsp*RIP93, *rsp*RIP94, *Rsp*RIP97, *Rsp*RIP100, *Rsp*RIP102, *Rsp*RIP103 and *rsp*$^{+}$, all in the demethylated condition, was used to perform dot blot analysis (as described in Materials and Methods), using *rsp*$^{+}$ as a probe. The intensity of the signal obtained for the different *Rsp*^{RIP} alleles was then normalized with the one obtained for the *rsp*$^{+}$ allele. When the percent sequence identity was plotted against the relative intensity, a strong linear correlation was obtained (r^2 = 0.9361, Figure 8A). Third, having validated the dot blot assay, we applied it to the analysis of all sequenced and unsequenced *Rsp*^{RIP} alleles. For this, we estimated the relative intensities for each allele, normalized it to wild-type, and plotted against its observed meiotic silencing in the absence of DNA methylation (Figure 8B). The moderate linear correlation obtained (r^2 = 0.8472), suggests that the number of point mutations contributes, at least partially, to the DNA methylation-independent behavior of the alleles in question. Note that this last linear correlation is similar to the one calculated when the percent sequence identities of the sequenced *Rsp*^{RIP} alleles relative

Table 1. Comparison of the mutational degree of different RIP alleles

Gene and Allele[a]	Number of Mutations[b]	Length of Duplicated Region	% ID to Wild-type[e]	RIP Index I[f]	RIP Index II[f]	Accession Number
rsp$^+$	0	NA[d]	100.0	0.74	1.19	AY290766
*rsp*RIP94	129	4392	97.2	0.99	1.02	AY313950
*Rsp*RIP93	254	4392	94.0	1.20	0.87	AY313949
*Rsp*RIP103	371	5260	92.8	1.17	0.89	AY582754
*Rsp*RIP102	309	5260	94.0	1.19	0.86	AY582753
*Rsp*RIP100	427	5260	91.7	1.24	0.83	AY582752
*Rsp*RIP97	465	5260	91.0	1.29	0.74	AY582751
sad-1$^+$	0	NA[d]	100.0	0.58	1.27	AY029284
*Sad-1*RIP64	462	2708	83.0	1.28	0.58	AF500110
am$^+$	0	NA[d]	100.0	0.68	1.15	K01409
*am*RIP8	162	2644	93.9	1.19	0.91	V32106
*Punt*RIP1	ND[c]	1874	ND[c]	1.31	0.57	AF181821

[a]genes names and alleles designations are: *roundspore* (*rsp*--recessive allele *or Rsp*—dominant allele), *suppressor of ascus-dominance-1* (*sad-1*), and *amination deficient* (*am*).

[b]Number reflects the number of mutations present in the duplicated region. For *Rsp* alleles a mutant base was only scored if the identity of that base was unambiguously determined across all *Rsp*-alleles sequenced.

[c]ND = Not Determined, because the sequence of the wild-type precursor is unknown.

[d]NA = Not Applicable.

[e]% Identity (% ID) is relative only to the wild-type duplicated region.

[f]RIP Index I = TpA/ApT. RIP Index II = (CpA + TpG)/(ApC + GpT). TpA/ApT and (CpA + TpG)/(ApC + GpT) are both RIP-indices described in (SELKER *et al.* 2003). Heavily RIP genes have higher TpA/ApT scores and lower (CpA + TpG)/(ApC + GpT) scores.

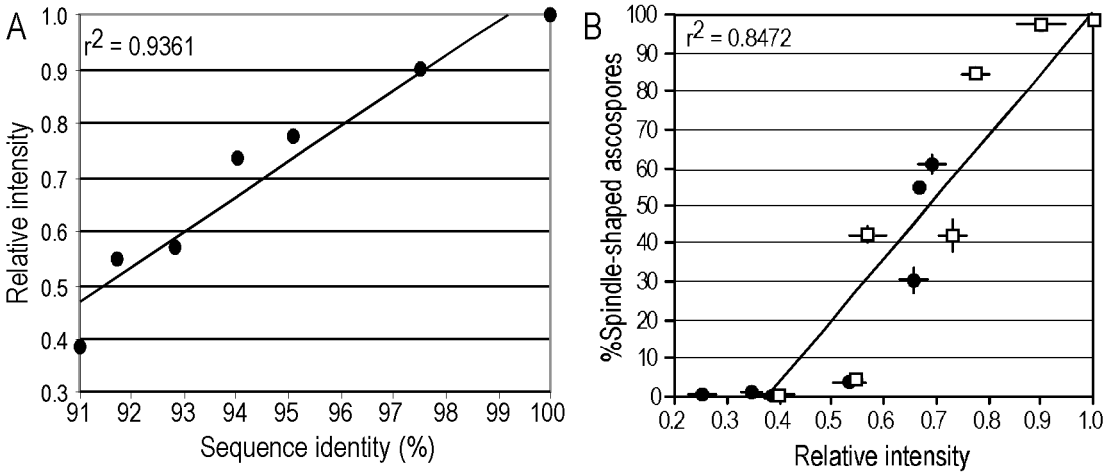

Figure 8. The meiotic dominance of *Rsp*RIP alleles moderately correlates with a stronger sequence divergence.A) Validation of Dot-Blot method for quantifying DNA sequence divergence. Relative intensity is plotted against percent sequence identity from sequenced alleles. The relative intensity was determined by measuring the signal obtained when DNA corresponding to each *Rsp*^{RIP} allele was immobilized on a Nitrocellulose membrane, and hybridized with radiolabeled fragments corresponding to the *rsp*$^{+}$ wild-type region. The probe corresponds to the 5,252 bp fragment containing the promoter and coding region of *rsp*$^{+}$ (coordinates 1123 to 6375) that was also used to obtain the new *Rsp*^{RIP} alleles. B) The meiotic dominance of *Rsp*^{RIP} alleles moderately correlates with a stronger sequence divergence. The percentage of spindle-shaped ascospores obtained for each *Rsp*^{RIP} allele plotted against their own relative intensity. The percentage of spindle-shaped ascospores was obtained from *dim-2* homozygous crosses between strains carrying the indicated *Rsp*^{RIP} allele and wild-type (*rsp*$^{+}$) strains. Black dots represent unsequenced alleles. Open squares represent sequenced alleles.

to wild-type, were plotted against their respective observed meiotic silencing in the absence of DNA methylation ($r^2 = 0.8527$).

The DNA methyltransferase gene (*dim-2*$^+$) may affect meiotic *trans*-sensing at other loci as well

To determine if the observed dependency on DNA methylation applies to other loci, we studied how the behavior of *Sad-1*^{RIP64}, a semi-dominant RIP-allele of *Sad-1* we previously isolated (Lee *et al.* 2003b), affected the silencing of the *rsp*$^+$ wild-type allele in *rsp*$^+$ x *Rsp*$^{\Delta(2)}$ heterozygous crosses (Figure 9). Here, the level of silencing induced by unpairing *rsp*$^+$ is constant and independent of DNA methylation (compare cross 1 with 2, Figure 9). Because the inability of *sad-1*$^+$ to pair with the *Sad-1*^{RIP64} allele induces partial silencing of the *sad-1*$^+$ wild-type allele itself (Lee *et al.* 2003b; Shiu *et al.* 2001), and given the absolute requirement of the SAD-1-RdRP for silencing, we reasoned that if in addition to unpairing *rsp*$^+$ (in a DNA methylation-independent manner), we were to unpair the *sad-1*$^+$ wild-type allele as well, the level of *rsp*$^+$ silencing would correlate with the level of *sad-1*$^+$ pairing, which in turn would be dependent on the level of DNA methylation of the *Sad-1*^{RIP64} allele. The dominance of the *Sad-1*^{RIP64} allele should then be directly proportional to its own level of DNA methylation, assuming DNA methylation does indeed affect the pairing of *sad-1*$^+$ with the *Sad-1*^{RIP64} allele. Since *sad-1*$^+$ silencing is expected to significantly decrease or eliminate the amount of *sad-1*$^+$ transcript and SAD-1 protein below that which is predicted for a single functional gene, a direct correlation between the dominance of *Sad-1*^{RIP64} and the level of *sad-1*$^+$ silencing was expected. Lowering the amount of SAD-1 protein should, in turn, translate in both lower silencing of *rsp*$^+$ and higher percentage of spindle-shaped ascospores.

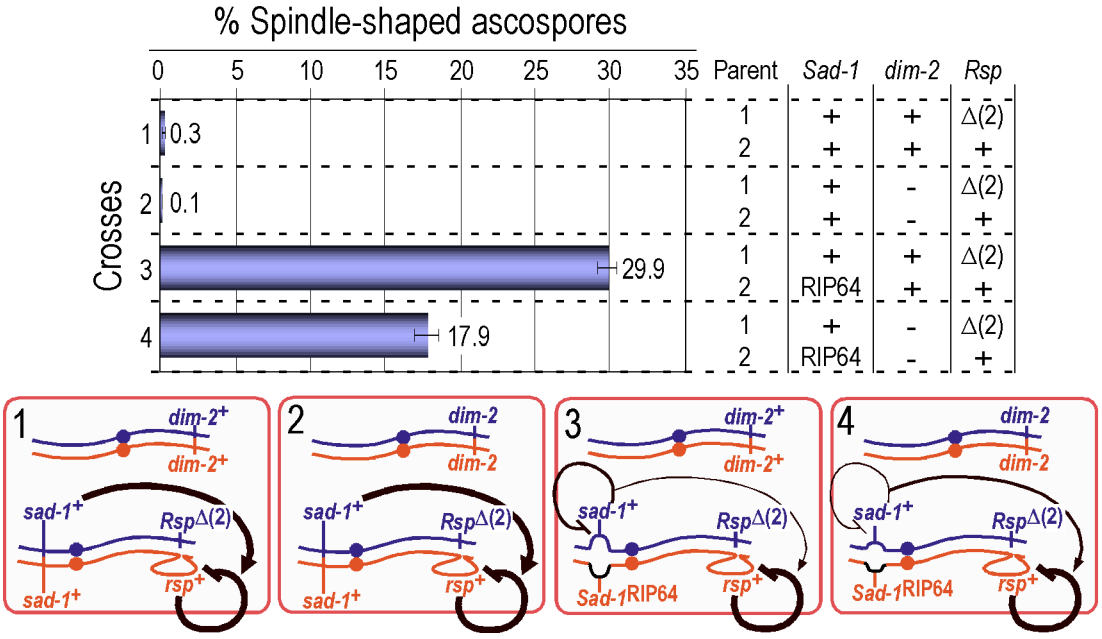

Figure 9. The meiotic dominance of *Sad-1*RIP64 is partially dependent on DNA methylation. The panel on top shows the percent of spindle-shaped ascospores obtained in crosses 1 to 4. Below, diagrams of diploid zygote cells corresponding to the different crosses are presented. The numbers inside the diagrams correspond to the cross numbers of the top plots. For simplicity, only one of the two sister chromatids is indicated, with the circles representing the centromeres. Relevant alleles and their relative position on the chromosomes (*dim-2* and *dim-2*$^+$ on LGVII and *sad-1*$^+$, *Sad-1*RIP64, *rsp*$^+$ and *Rsp*$^{\Delta(2)}$ on LGI) are marked. The thickness of the arrow-headed lines directly represent the predicted relative levels of SAD-1 enzyme activity during meiotic silencing. Similarly, the thickness of bar-headed lines directly represents the predicted relative levels of unpairing (or failure of *trans*-sensing) of the different alleles. The dominance of *Sad-1*RIP64 was assayed by determining the level of silencing of *rsp*$^+$ in crosses between *rsp*$^+$ and *Rsp*$^{\Delta(2)}$ alleles, in the presence (*dim-2*$^+$) and absence (*dim-2*) of DNA methylation. Since *sad-1*$^+$ is required for silencing *rsp*$^+$, the higher the dominance of the *Sad-1* alleles, the higher the predicted unpairing of the *sad-1*$^+$ wild-type allele, which in turn would result in more *sad-1*$^+$ gene silencing and less *rsp*$^+$ gene silencing. Each number represents an average of at least four crosses with a mean of 1,496 ascospores per cross.

This prediction was confirmed, in *sad-1*$^{+}$ x *Sad-1*^{RIP64} heterozygous crosses, the degree of *rsp*$^{+}$ silencing was lower in the presence of DNA methylation (compare cross 3 with 4, Figure 9). Consistent with what we observed before, meiotic silencing was unaffected by the loss of genome methylation (cross 2, Figure 9, and Figure 4B). These data are consistent with the proposal that DNA methylation, directly or indirectly, interferes with meiotic *trans*-sensing of all methylated alleles, but not with meiotic silencing.

DISCUSSION

DNA methylation affects meiotic *trans*-sensing but not meiotic silencing

In this work, we have arguably genetically dissected meiotic *trans*-sensing from meiotic silencing by demonstrating that DNA methylation affects the dominance of RIP alleles in meiosis without generally interfering with meiotic silencing. This observation assigns methylated DNA regions a previously undetected role in chromosome sensing. We also determined that DNA sequence is an important parameter considered in the process.

Our observations are consistent with the existence of two mechanisms, meiotic *trans*-sensing and silencing, operating jointly in meiosis. During meiosis chromosomes “sense” the identity of the regions they are supposed to pair with in the homologous chromosome. If the regions are equivalent, development proceeds normally. If not, the meiotic *trans*-sensing machinery recognizes the unpaired region and activates meiotic silencing. The *trans*-sensing step must be exquisitely sensitive given that even the methylation status of the alleles compared is taken into account in the determination of their pairing potential (*i.e.,* DNA methylation, directly or indirectly, inhibits this meiotic *trans*-sensing).

The model predicts that meiotic *trans*-sensing can be dissociated from meiotic silencing. It also predicts that the strength of meiotic silencing is dependent upon

the degree of unpairing. Previously, we demonstrated that the larger the region of DNA that is unpaired, the greater the level of meiotic silencing of a reporter gene that is observed (Lee *et al.* 2004). Confirming these observations, crosses between *sad-1*$^{+}$ with different *Sad-1* alleles result in different levels of SAD-1 enzyme, which in turn, directly affect the level of meiotic silencing (Shiu and Metzenberg 2002; Shiu *et al.* 2001).

Why do RIP alleles affect meiotic *trans*-sensing?

Some time ago, Colot *et. al*. (1996), made the amazing observation that the DNA methylation state of an allele could be transferred to its homolog during meiotic chromosome pairing by a mechanism related to gene conversion. This observation suggests a mechanism by which a RIP allele could potentially transfer its state to its partner on the opposite chromosome. If this were the case, then the "silencing" we observe could be due, at least in part, to this epigenetic transfer. Three pieces of evidence strongly argue against this: First, all *Rsp*$^{\mathrm{RIP}}$ alleles tested, can be suppressed entirely by *Sad-1*$^{\Delta}$. If even a fraction of the silencing observed would be due to this proposed "epigenetic transfer," this fraction would presumably not be suppressed by the *Sad-1*$^{\Delta}$ allele. We would have detected this difference. Second, the epigenetic transfer would have to be unidirectional and highly efficient to be the primary silencing mechanism. The efficiency of transfer reported by Colot *et al.* (1996) was low. Third, the maintenance of this "epigenetic transfer," would have to be transient and confined only to ascus development, since the segregation of RIP phenotypes we observe among the progeny is Mendelian, unless there is a mechanism for erasing the transfer only from the newly silenced allele.

This work was possible due to the observation that, while highly identical to its wild-type allele, *Rsp*$^{\mathrm{RIP93}}$ clearly shows a dominant genetic behavior in the presence of DNA methylation, versus a semi-recessive genetic behavior in its

absence. Why would this be? RIP alleles differ from their wild-type counterparts in essentially four ways: First, they contain point mutations. Second, they tend to be methylated. Third, they can be associated with trimethylated histone H3 lysine-9 (H3-K9) containing chromatin. Fourth, they are generally transcriptionally inactive. In the absence of DNA methylation (*e.g.,* as when *Rsp*RIP93 is propagated in a *dim-2* mutant background), some of these differences can be erased. The DNA methylation is always lost (Kouzminova and Selker 2001). The H3-K9-trimethylated mark has also been observed to disappear at some loci (*e.g., am*RIP8), but not at others (*e.g., Punt*, see Table 1) (Tamaru and Selker 2001; Tamaru and Selker 2003; Tamaru *et al.* 2003). Finally, the transcriptional elongation that is highly perturbed in the presence of DNA methylation, can be restored in its absence, at least for some loci (Rountree and Selker 1997). In this work, we clearly show that DNA methylation is one of the parameters responsible for the dominance displayed by some of the RIP alleles here studied. We also show a correlation between DNA identity and dominance. These observations, however, do not discard the possibility that other factors, like chromatin state, might play a role in the sensing step.

But what is involved in homolog *trans*-sensing? Or how do chromosomal regions evaluate their degree of equivalence with opposite regions? We think that at least two different, but potentially interrelated mechanisms are involved: DNA identity and chromatin identity. The first model is simple and attractive and can potentially explain all our observations. It considers that DNA identity is the only parameter considered during chromosome sensing. Under this model, a 5-methylcytosine (5-mC) base is “seen” as a “fifth base.” This is, to a guanine, 5-mC would be as different as an adenine. It follows that the level of DNA methylation of a given chromosomal region will determine its degree of perceived identity when compared to an equivalent wild-type region. For example if the identity between a demethylated *Rsp*RIP93 and *rsp*+ allele is

94%, in the presence of DNA methylation, their identity would be 55%, assuming all cytosines become methylated. This model is supported by the moderate correlation we detected between sequence identity and dominance in the absence of DNA methylation.

The other, non-mutually exclusive, chromatin identity model states that the meiotic *trans*-sensing machinery determines the pairing potential of two opposite regions at the level of chromatin. Two euchromatic or two heterochromatic regions would be considered homologous. In contrast, if a euchromatic region on one chromosome is compared to a heterochromatic region on the other, the regions would be considered heterologous and meiotic silencing would be triggered. Under this model the observed variance in the dependence on DNA methylation for allele dominance can be explained by the different abilities of the alleles to maintain heterochromatin in the absence of DNA methylation. Similarly, other loci also differ in their maintenance of trimethylated H3-K9 in a *dim-2* background (Tamaru *et al.* 2003). Furthermore, the correlation detected between sequence identity and dominance could be superficial, reflecting more a difference in AT-richness as opposed to simply changes in nucleotide identity. Given that the AT-richness of a region correlates with *de novo* DNA methylation (Tamaru and Selker 2003) and that DNA methylation requires trimethylated H3-K9 (Tamaru and Selker 2001), it is possible that the *Rsp*$^{\mathrm{RIP}}$ alleles we isolated differ in their ability to recruit the silencing complexes that recognize this AT-rich signature, as described in the model proposed by Selker, *et. al.* (2002). Given that RIP mutates CpA dinucleotides preferentially, the mutation level of a DNA region can be easily predicted by using two RIP indexes (TpA/ApT and [CpA+TpG]/[ApC+GpT], Table 1) (Selker *et al.* 2003). If the silencing complexes that recognize and methylate, demethylated RIP regions *de novo* also recognize qualitatively this preference, alleles with strong RIP indexes would also be predicted to maintain silencing better in the absence of DNA methylation.

This latter model could also explain the anomalous behavior of the *Rsp*RIP102 and *Rsp*RIP103 alleles. Based on DNA identity alone, *Rsp*RIP103 should be more dominant than *Rsp*RIP102 (*i.e.,* 92.8% versus 94.0%, Table 1). Biologically though, both alleles have statistically identical levels of dominance (Figure 6). It is therefore possible that both of these alleles maintain similar levels of heterochromatin. Perhaps the quality of the AT-rich regions present in *Rsp*RIP102 is more successful at recruiting heterochromatin than those present in *Rsp*RIP103. The RIP indexes calculated for *Rsp*RIP102 and *Rsp*RIP103 are consistent with this idea (Table 1). Similar arguments can also explain the drastic differences in dominance observed for *Rsp*RIP103 and *Rsp*RIP100 alleles (Figure 6). The difference in percent identity to wild-type of these last two alleles is similar to the difference in percent identity to wild-type of *Rsp*RIP102 and *Rsp*RIP103 (*i.e.,* 1.1 [92.8 – 91.7] versus 1.2 [94.0 – 92.8], Table 1).

Could *trans*-sensing be mechanistically related to recombination?

DNA methylation is known to inhibit meiotic recombination in Ascobulus (Maloisel and Rossignol 1998), and can interfere with V(D)J recombination in mammals (Engler and Storb 1999). Chromatin structure is also known to affect the positioning of the double strand breaks (DSB) associated with recombination (Wu and Lichten 1994). Sequence homeologies are established barriers for DNA recombination in a mismatch-repair-dependent manner (Hunter *et al.* 1996; Radman and Wagner 1993; Rayssiguier *et al.* 1989). In *Saccharomyces cerevisiae*, recombination inhibition may occur both by an inhibition of DSB formation at sequence heterology (Rocco and Nicolas 1996; Xu and Kleckner 1995), and by rejection of heteroduplex DNA in homeologous regions (Alani *et al.* 1994; Chambers *et al.* 1996; Hunter *et al.* 1996). It is therefore possible that the inhibition of recombination associated with the DNA methylation and/or sequence homeology of RIP alleles triggers meiotic silencing. If this is the case,

then recombination may promote or facilitate meiotic *trans*-sensing, which in turn reduces meiotic silencing. This model predicts that the “sensing” of recombination-deficient chromosomal regions is impaired and that meiotic silencing might just not occur in these neighborhoods. This model also predicts that regions that are recombination proficient might be more sensitive to being unpaired. Finally, the model predicts that, at least some components of the meiotic recombination apparatus might be part of the meiotic *trans*-sensing machinery.

Consistent with this, meiotic silencing is partially responsible for the meiotic sterility of Neurospora interspecific crosses (Shiu *et al.* 2001), an effect similar to the one seen in *S. cerevisiae,* where the anti-recombination effects of mismatch repair leads to meiotic sterility in interspecific crosses (Chambers *et al.* 1996; Hunter *et al.* 1996). In maize, a role for Rad51/RecA in chromosome homology recognition during meiosis has been postulated (Pawlowski *et al.* 2003), and male mice defective for the components of the DNA mismatch repair recognition system, exhibit abnormal chromosome synapsis in meiosis (Baker *et al.* 1995).

In *S. cerevisiae*, accurate pairing of homologous chromosomes requires Hop2, a protein implicated in homology searching and/or recognition. Mutants in this meiosis-specific recombinase show promiscuous pairing between non-homologous chromosomes (Leu *et al.* 1998), a defect that can be partially suppressed by over-expressing the recA homolog Rad51 (Tsubouchi and Roeder 2003). Dmc1, a Rad51 paralog, works with Rad51 and Hop2 to ensure the legitimacy of the pairing (Tsubouchi and Roeder 2003). Both Hop2 and Dmc1 could not be detected by BLAST searches of the current assembled Neurospora genome (Borkovich *et al.* 2004). Therefore, if *trans*-sensing is mechanistically related to meiotic recombination, as we propose here, a different mechanism must exist for homology searching and/or recognition in Neurospora.

Alternatively, this function could be fulfilled by the sole Neurospora Rad51 homolog MEI-3. It is noteworthy that, like Neurospora, *Caenorhabditis elegans* lacks *dmc1* and silences unpaired DNA in meiosis (Bean *et al.* 2004).

Is meiotic *trans*-sensing relevant?

Trans-sensing phenomena are relevant to human disease. Pairing abnormalities result in homologue non-disjunction events (*i.e.*, aneuploidy), a major cause of spontaneous abortion and developmental defects in humans, such as Down's syndrome (Hassold and Hunt 2001). Normal development and adult phenotype requires normal imprinting (*i.e.*, the tissue- and timing-specific functional haploidy) of specific human genes (Bennett *et al.* 1997; Constancia *et al.* 1998; Hall 1997), and all imprinted regions studied to date consistently show pairing (Lalande 1996; LaSalle and Lalande 1996; Riesselmann and Haaf 1999). Pairing-dependent genetic phenomena have long been known to occur in *Drosophila*, where homologue pairing influences gene expression (Henikoff and Comai 1998; Henikoff *et al.* 1995; Pirrotta 1999; Sass and Henikoff 1999; Wu and Morris 1999). They have also been postulated to play important gene regulatory roles in the somatic and meiotic cells of plants (Chandler *et al.* 2000; Chandler and Stam 2004; Matzke *et al.* 2001; Stam *et al.* 2002). In addition, some genes expressed during meiosis and development in the mouse have also been postulated to *trans*-interact with each other (Duvillie *et al.* 1998; Herman *et al.* 2003). Paramutation in maize (Chandler *et al.* 2000; Stam *et al.* 2002), transvection of *brown*Dominant (bw^D) in Drosophila (Sass and Henikoff 1999), and the transvective-like effects observed between *lox* recombination sites in the mouse (Rassoulzadegan *et al.* 2002), are all similar to the dominance of RIP alleles in three ways: First, they could represent sensing-dependent phenomena, in which differential DNA methylation or heterochromatin between the loci might be associated with the silencing of the normal allele. Second, these phenomena, with the possible exclusion of the observation in mice, seem

to depend on successful sensing to silence the homologous allele where as meiotic silencing seems to depend on the failure of sensing to silence the homologous allele. Third, these phenomena seem to involve changes in epigenetic state of the normal allele similar to what is seen in Ascobulus, rather than post-transcriptional silencing. Importantly, while the output of this *trans*-sensing may be different, the mechanism of *trans*-sensing may be universally shared.

MATERIALS AND METHODS

Procedures for Southern blot analysis, and other nucleic acid manipulations were performed as described (Pratt and Aramayo 2002). Similarly, growth conditions, conidial spheroplast preparation and fungal transformation were performed as described (Pratt and Aramayo 2002). Homokaryon purification was performed as described (Lee *et al.* 2003a; Pratt and Aramayo 2002). The formulas for the Vogel's Medium N, the Westergaard's Medium, and the sugar mixture of Brockman and de Serres have been described by Davis and de Serres (1970).

Construction of *Rsp*$^{\Delta}$ and isolation of *Rsp*^{RIP} alleles

The construction of the *Rsp*$^{\Delta(2)}$ allele and of the *Rsp*^{RIP93} and *rsp*^{RIP94} alleles (Figure 4A), will be described elsewhere. The new series of *Rsp*^{RIP} alleles was obtained as follows: Strain DLNCT115 (Table A1) containing a 5,252 kbp fragment of *Rsp* (coordinates 1123-6375, Figure 4A) inserted at the *histidine-3* (*his-3*) locus in a *Rsp*$^{\Delta(1)}$ background was crossed to RPNCR75A to obtain RPNCR92A, a *dim-2* strain containing both the insert and a *rsp*$^{+}$ wild-type locus. The *Rsp* locus was then mutagenized by RIP, by crossing RPNCR92A to RPNCR75A. Histidine-requiring progenies were then used to fertilize each of six fluffy tester strains. The first two tester strains (RPNCR43A and RPNCR44A) tested for *Rsp* loss-of-function. The second two tester strains (RANCR49A and

RANCR50A) tested for dominance/recessivity of the *Rsp*^{RIP} alleles in the presence of DNA methylation. Dominant *Rsp*^{RIP} alleles shot round spores under these conditions. The last two testers (RPNCR45A and RPNCR46A) tested for dominance/recessivity in the absence of DNA methylation.

Sequencing of *Rsp*^{RIP} alleles

Rsp^{RIP} alleles were amplified by PCR from genomic DNA using oligonucleotides ORP115 and ORP116. The sequence of these and all other oligonucleotides used in this work are given in Table B1. The product of this reaction was then used as a template for a nested PCR reaction using oligonucleotides ORP113 and ORP114. The product of this second PCR reaction was digested with *Not*I and cloned into the *Not*I site of pRATT11c (Pratt and Aramayo 2002). Clones were then sequenced using both the GeneJumper Primer Insertion Kit (Invitrogen, Carlsbad, CA, USA) and the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (PEBiosystems, Foster City, CA, USA). Sequences were generated on an Applied Biosystems Model 377 or 373 automated DNA sequencer at GeneTechnologies Laboratory (Institute of Developmental and Molecular Biology—IDMB, Texas A&M University, College Station, TX, USA). Gaps and ambiguous bases made up no greater than 1% of the finished sequence. In determining percent identity, gaps and ambiguous bases in any of the allele sequences were ignored in all sequences (we had a total of 88 unscored bases). Percent sequence identities in Table 1 are relative to the wild-type *Eco*RI to *Cla*I region diagrammed in Figure 4A (coordinates 1123 to 6375). The GenBank Accession Numbers corresponding to the *rsp*$^{+}$, *Rsp*^{RIP93}, *rsp*^{RIP94}, *Rsp*^{RIP97}, *Rsp*^{RIP100}, *Rsp*^{RIP102}, *Rsp*^{RIP103} alleles are cited in Table 1.

Isolation of *dim-2*^{RIP} alleles

A 1.2-kbp PCR fragment spanning part of the catalytic site of *dim-2* was amplified from genomic DNA of a *dim-2*$^{(1)}$ containing strain with the following oligonucleotides: ORP083 and ORP084. This was cloned into the *Nru*I site of pRATT11c (Pratt and Aramayo 2002) A 965 bp *Eco*RI/*Bgl*II fragment containing the *dim-2* region was then subcloned from this plasmid into the *Eco*RI/*Bam*HI sites of the *his-3* insertion vector pJHAM3 (Haag and Aramayo 2003), to yield pRATT47. *dim-2* was duplicated by transforming DLNCR99A with pRATT47 to yield RPNCT88A. Homokaryons were obtained by 5-fluorodeoxyuridine selection as described (Lee *et al.* 2003a). RPNCT88A was then crossed to DLNCR93 to RIP the *dim-2* duplication. Tall (*Rsp*$^{\Delta}$ has a stunted phenotype) and *his-3* (lacking the duplication) progeny were screened for loss of methylation at *Rsp*^{RIP93} by Southern blot with *Bam*HI digested DNA. Two strains showed a loss of DNA methylation, RPNCR89A and RPNCR90A, which contain *dim-2*^{RIP89} and *dim-2*^{RIP90} respectively. RIP was confirmed by sequencing the alleles.

DNA analysis

For each strain, a starter cultures was grown in 1 ml of supplemented liquid Vogel's medium (Davis and de Serres 1970) at 35°C and allowed to conidiate at 25°C. The mycelia mats from each starter culture were then transferred to a 250 ml Erlenmeyer flask containing 20 ml of supplemented liquid Westergaard's medium. Cultures were grown for 5.5 days at 25°C in constant light with periodic mixing to minimize aerial growth. DNA was extracted as described (Pratt and Aramayo 2002).

Dot blots were made using a Minifold dot blot apparatus (Schleicher and Schuell, Keene, NH, USA). DNA from each strain was spotted in triplicate on Nytran membranes (Schleicher and Schuell). Membranes were then hybridized

with ^{32}P-dCTP radiolabeled DNA fragments obtained by random priming extension of the *rsp*$^{+}$ DNA region as described (High Prime DNA labeling kit [RocheMolecular Biochemicals, Mannheim, Germany]). After hybridization, the blots were washed at 75°C with 2X SSC and exposed. X-ray films were scanned at 600 dpi and the net intensity of the dots was quantified using the Image Analysis software Kodak 1D 3.5.3 (Eastman Kodak Company, New Haven, CT, USA). Small differences in loading were corrected for each dot by using relative intensities when probed with β-tubulin (*benomyl* [*bml*]). The signals obtained for each of the three sets of spots were corrected and normalized independently. The relative intensity of each dot was calculated by dividing the intensity of each dot by the intensity of the *rsp*$^{+}$ wild-type dot for that set. A strip containing dots with 0.5, 1.0, 2.0 and 4.0 X volume of DNA was probed in conjunction with the *Rsp* alleles to ensure that the measured intensities lay in a linear range.

Strain construction

The description of the strains used in this study and the methods of their construction are given in Table A1. The *dim-2* alleles were determined by Southern blots of genomic DNA digested with *Bam*HI or with *Bam*HI/*Eco*RI probed with radiolabeled DNA fragments obtained by random priming extension (obtained as described [High Prime DNA labeling kit (RocheMolecular Biochemicals)]), corresponding to either the *Rsp* region or the *zeta-eta* region. The *Rsp* locus has a normally methylated *Bam*HI site located upstream (data not shown). The state of the *Rsp* alleles were determined by analyzing the Southern blots probed with the *Rsp* region or by crossing the strains to testers RPNCR43A or RPNCR44A (Table A1).

Genetic crosses—Set up and scoring

Partners were co-inoculated in a Petri dish containing Westergaard's medium, and incubated at 25°C for six to eight days. The two co-inoculated strains

usually meet after day three to five. Excess conidia were removed only when fertilization was evident. Crosses started shooting ascospores approximately 14 days after inoculation. Spores were harvested no sooner than 25 days after point inoculation. When one of the crossing partners carried the *fluffy* (*fl*) mutation, conidia from the *fl*$^{+}$ partner were used as males, fertilizing the female structures in the lawn of *fl* strains.

Importantly, *Rsp* mutants mostly ooze their progeny through the perithecial ostiole. The remaining spores are weakly shot and most of them never reach the lid of the Petri Dish. Therefore, to accurately assess the degree of silencing, spores must be collected from both, the lids and the surfaces, of the Petri dishes. For this, we flooded the plate with sterile water and scrapped the perithecia off the surface of the agar with a sterile glass rod. The slurry of spores and tissue was then used to harvest the spores from the lid of the plate. The combined slurry was then transferred into a centrifuge tube. The process was repeated; this time washing both the front and back of the agar. After centrifugation, the supernatant was carefully removed by aspiration and discarded. Aliquots of the suspension were transferred onto glass microscope slides and under a cover slip. The degree of silencing was determined by taking nine pictures from different random fields (11X magnification + 115 mm zoom). Pictures were printed and the number of round and spindle-shaped spores was determined.

While we observe that crosses between the same parents vary in their absolute number of wild-type ascospores produced (within ± 10% range), the ratio of wild-type ascospores produced by a given allele (e.g., *Rsp*$^{\mathrm{RIP93}}$) relative to the one produced by another allele (e.g., *Rsp*$^{\mathrm{RIP105}}$) is maintained relatively constant within the same experiment. Why this variability occurs is not clear, but we believe is due to the contribution of several, perhaps overlapping factors, which

include but are not restricted to: temperature, oxygen, lunar phase and illumination. If any or all these factors, directly or indirectly, influence DNA methylation, they would also influence the dominant behavior of the *Rsp*^{RIP} allele in question. This is why all statements regarding the behavior of one particular allele compared to another allele have been made based on data obtained from crosses that were set up and harvested at the same time.

Contributions

All of the data presented in this chapter has been published (Pratt *et al.* 2004). Some of the text of this publication has been modified to hopefully increase clarity, to reduce redundancy of information between the chapters, and to increase consistency in terminology between chapters of this dissertation. Dong Whan Lee cloned the *Rsp* reporter and created and characterized the *Rsp*^{RIP93}, *rsp*^{RIP94}, *Rsp*^{ect} and *Rsp*Δ(2) alleles (Figure 4A), and also contributed several of the strains for this study (DLNC strains, Table A1). Robert J. Pratt designed and performed all other experiments and interpreted the data presented. Rodolfo Aramayo advised on aspects of the experimental design and implementation and on the interpretation of the data. RJP and RA wrote the published manuscript.

CHAPTER III
SPO11-DEPENDENT RECOMBINATION AND SYNAPSIS ARE DISPENSABLE FOR MEIOTIC *TRANS*-SENSING AND MEIOTIC SILENCING

INTRODUCTION

Meiosis, the core engine of sexual reproduction, is a complex process that results in the production of recombinant haploid genomes. During this specialized mode of cell division, chromosomes perform an amazingly orchestrated dance composed of pre-meiotic DNA replication, Meiosis I and Meiosis II, all designed to ensure their proper segregation (Zickler and Kleckner 1998; Zickler and Kleckner 1999). Meiosis in Neurospora occurs in a complex developmental structure, the perithecium, where zygotic cells undergo meiosis in an unsynchronized manner. The zygote, or ascus mother cell, is the only diploid cell known in this organism. Here, meiosis and the subsequent post-meiotic mitosis occur. It is also inside the ascus cell that the resulting eight nuclei undergo cellularization (i.e., ascospore formation). After cellularization is complete melanized ascospores are ejected from the perithecium (Raju 1980; Raju 1992).

In Neurospora, pre-meiotic DNA replication occurs prior to karyogamy (i.e., nuclear fusion). Once nuclei of opposite mating types fuse, replicated chromosomes (now composed of two sister chromatids held together by cohesin) initiate the early stages of meiosis. Although the molecular processes that occur early in meiosis are poorly understood, we know that chromosomes first find their respective homologues and then proceed to evaluate the molecular identity of their pairing partners. As chromosomes condense and initiate the leptotene stage of Meiosis I, programmed double-stranded breaks (PDSB) are introduced, and homologous chromosomes stably align and participate in long-distance pairing. The beginning of the zygotene stage is

marked by synapsis, or short-distance pairing, and initial formation of the proteinaceous synaptonemal complex (SC) structure between homologous chromosomes. The formation of bivalent chromosomes marks the beginning of the pachytene stage. Originally, difficult to see because of the small nuclear size, as the nucleus gradually enlarges and the ascus cell grows, bivalent chromosomes become more elongated and better spread. Here, paired chromosomes contain a complete SC along their length. In many organisms, failure to resolve recombination intermediates at this stage results in activation of the pachytene checkpoint (PCH) of meiosis and meiotic arrest (Roeder and Bailis 2000). If the PCH is passed, the SC is dissolved and chromosomes decondense at diplotene, followed by re-condensation at diakinesis and separation of homologous chromosomes. Meiosis II, a second, mitosis-like division that separates sister chromatids without DNA replication then occurs, followed by a post-meiotic mitosis, giving rise to an ascus with a linear array of eight spores in an order that reflects their lineage. In Neurospora, many (200-400) asci undergo meiosis asynchronously within a fruiting body called the perithecium and remain attached to one another as a cluster of tissue called the rosette (Page and Hawley 2003; specifics for Neurospora reviewed Raju 1980; Raju 1992; meiosis in general reviewed Zickler and Kleckner 1999).

In Neurospora, meiotic *trans*-sensing is quite sensitive. For example, silencing of the *Ascospore maturation-1* (*Asm-1*) reporter gene can be detected by unpairing DNA fragments that are as small as 1.5-kbp (Lee *et al.* 2004). This is equivalent to detecting an aberration that is <0.02% of the length of the chromosome carrying it. Importantly, deletions or insertions are not the only kind of aberration detected. Partially homologous (i.e., homeologous) regions can also activate meiotic silencing, as it was demonstrated for alleles of a second reporter gene *Round spore* (*Rsp*) (Pratt *et al.* 2004). In this case, a series of alleles generated by RIP were shown to efficiently activate meiotic silencing, even though the

sequence identity of some of these alleles to wild type was as high as 94%. In addition, these experiments also established that one of the factors taken into consideration during *trans*-sensing is the methylation state of the alleles being compared (Pratt *et al.* 2004).

If chromosomal regions are compared at this level of detail, it is tempting to hypothesize that meiotic *trans*-sensing would be done in conjunction with the intimate processes of recombination and synapsis. Mounting evidence supported a connection. First, *trans*-sensing is not simply a counting phenomenon; it is a chromosome-pairing phenomenon. Merely having one copy of the gene from each parent is not sufficient, the copies need to pair at allelic positions on homologous chromosomes (Aramayo and Metzenberg 1996). In yeast and mice, the link between recombination and chromosome pairing has been solidly established (Liebe *et al.* 2006; Petukhova *et al.* 2005; Petukhova *et al.* 2003; Pezza *et al.* 2006; Tsubouchi and Roeder 2003). Second, the indels and mismatches that trigger meiotic silencing can be detected in the heteroduplex DNA of recombination intermediates during meiosis by the DNA mismatch-repair (MMR) machinery (Harfe and Jinks-Robertson 2000; Kearney *et al.* 2001). Gene conversion frequency of a reporter does not approach the efficiency of meiotic silencing (Mitchell 1966; Srb *et al.* 1973). However, gene conversion only represents one of two outcomes of detection by MMR, repair or anti-recombination (i.e. abortion of the recombination attempt), and thus gene conversion frequency is likely an under-representation of MMR detection frequency (Harfe and Jinks-Robertson 2000). Third, both meiotic *trans*-sensing and recombination are sensitive to DNA methylation (Engler and Storb 1999; Maloisel and Rossignol 1998). Finally, in mouse meiosis, both recombination and the silencing of unpaired DNA are closely correlated with synapsis (Mahadevaiah *et al.* 2001; Turner *et al.* 2006).

To test whether *trans*-sensing depends on recombination and/or synapsis, we determined the extent of meiotic silencing in several meiotic mutants of Neurospora. We conclude that in Neurospora, synapsis and silencing are not related and demonstrate that the meiotic arrest observed in *Sad-1* mutants is not dependent on the presence of PDSBs. If detection of unpaired DNA occurs using the same basic molecular mechanism in all organisms, our results raise the possibility that detection of unpaired DNA might be universally independent of the machineries of stable homologue pairing and meiotic recombination.

RESULTS

Experimental rationale

To test the involvement of recombination in meiotic *trans*-sensing and meiotic silencing, we assayed meiotic silencing in several mutants of Neurospora. We first selected three genes whose loss of function in budding yeast results in meiotic dysfunction yet still allows production of some ascospores: *sporulation11* (*spo11*), *superkiller8* (*ski8*), *MutS homolog4* (*msh4*). We then constructed the corresponding loss-of-function mutants in Neurospora. In addition, we also used three previously characterized meiotic mutants of Neurospora: *meiosis-1* (*mei-1*), *Meiosis-2* (*Mei-2*) and *meiosis-3* (*mei-3*). In homozygous crosses, these mutants show different degrees of sporulation. For this reason, we used two kinds of reporters: a morphological reporter gene, *Rsp*, which can be evaluated by the formation of round ascospores when unpaired and silenced, as opposed to spindle-shaped ones when not silenced. We also used a non-morphological reporter gene, fusions of *histone H1* to *gfp* (*hH1::gfp*), which can be evaluated by the absence of a fluorescent signal in the unpaired and silenced, as opposed to its presence when not silencing. The use of *gfp* allowed us to test meiotic silencing in mutants that can initiate meiosis, regardless of their ability to produce ascospores; whereas, the use of *Rsp* served as a well-characterized

standard for meiotic silencing, but was only useful for mutants that still produced ascospores.

To inactivate *spo11*, *ski8* and *msh4*, we used RIP mutagenesis (Materials and Methods), in which Neurospora efficiently inactivates genes by introducing numerous GC-AT point mutations and frequently methylating and transcriptionally silencing the gene. RIP is a very useful method for obtaining mutant alleles of genes required for aspects of meiosis, since it can give recessive loss of function alleles (e.g., *spo11*$^{\text{RIP294}}$), unlike deletions or even strong RIP alleles (e.g., *Msh4*$^{\text{RIP174}}$), which can be dominant in meiosis. Such dominance makes it difficult to move the allele into different backgrounds through crosses without a suppressor of meiotic silencing present. All of the RIP alleles likely result in loss of function, because they contain multiple missense mutations and all except *spo11*$^{\text{RIP295}}$ contain more than one premature stop codon (Figure 10A-C). The degree to which function of allele *spo11*$^{\text{RIP295}}$ is lost is certainly questionable since it lacks a premature stop codon. However, silencing was also assayed in a cross using only the truncated *spo11*$^{\text{RIP294}}$ allele with the same results (below).

The rosette phenotypes of the *spo11* mutants mirrored those reported for other *spo11* mutants of Neurospora (Bowring *et al.* 2006). Homozygous crosses resulted in few asci with ascospores compared with wild-type (Figure 11A&B Vs Figure 11C&D). The asci produced always contained fewer than eight mature dark ascospores and frequently contained pale ascospores (Figure 11A&B), likely attributable to the observed differences in DNA content among spores in the asci (data not shown; (Bowring *et al.* 2006). The previously published study reported that 50% of pigmented ascospore were viable and that unpigmented

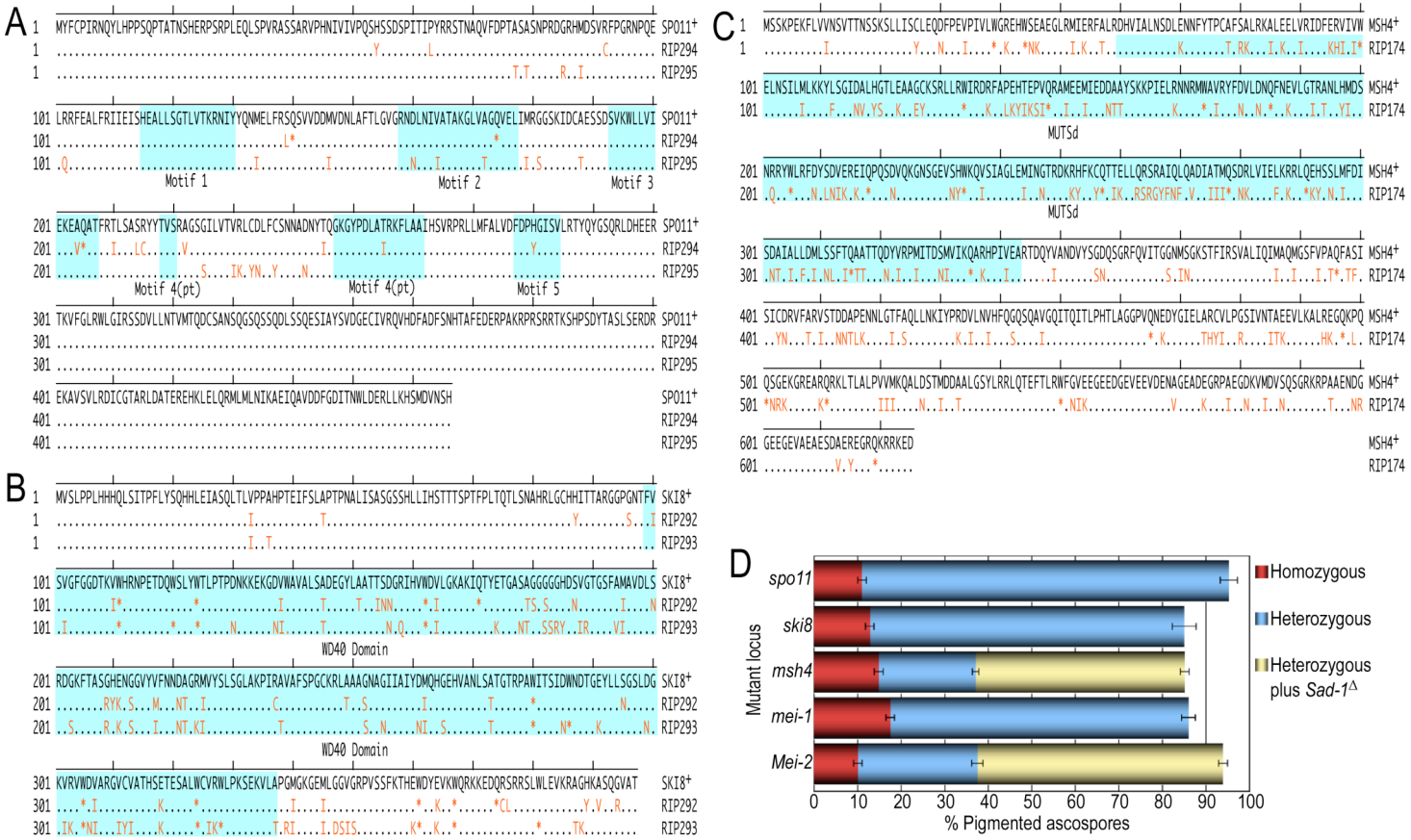

Figure 10. Characterization of ascospore-producing mutants of meiosis. A-C) Protein sequence changes introduced by RIP. The upper row is the wild-type sequence. Amino acids changed by RIP are indicated by the letter symbol of the amino acid substitution. Stop mutations are indicated as asterisks. A) *Spo11* alleles. Highlighted motifs were defined by Diaz *et al.* (2002) and the sequence is based on Bowring *et al.* (2006) B) *Ski8* alleles. The WD40 domain is highlighted. C) The *msh4* allele. The MUTSd domain is highlighted. Sequence is based on Conway *et al.* (2006). D) Sporulation defects in crosses with mutant loci. Pigmented ascospores can be viable but unpigmented ascospores are inviable. Crosses were either homozygous for the mutation (red) or heterozygous for the mutation (blue and yellow). *Msh4* and *Mei-2* were semi-dominant as evident by the low frequency of pigmented ascospores in heterozygous crosses (blue). Crosses heterozygous for these mutations and also heterozygous for *Sad-1*$^{\Delta}$ (yellow) resulted in higher percentages of pigmented ascospores. For the other loci, there were no significant differences between heterozygous crosses with or without *Sad-1*$^{\Delta}$, and the data for these crosses were combined (blue). See Table 2 for crosses.

ascospores, making up 90% of the total, were invariably dead (Bowring *et al.* 2006). All of our ascospore-producing mutants also produced high percentages of unpigmented ascospores, likely due to defects in chromosome segregation (Figure 10D). Together, the molecular data and the meiotic phenotypes strongly suggest that all the alleles tested represent loss of function.

Spo11-dependent programmed double-strand breaks are dispensable for meiotic silencing

The product of the *spo11* locus, the SPO11 protein, is a highly conserved transesterase that catalyzes the formation of the PDSBs that serve as the initiating substrate for meiotic recombination during the leptotene stage of meiosis (Keeney 2001; Krogh and Symington 2004). In fungi, Spo11 is required for significant stable long-distance pre-synaptic homologue co-alignment, synapsis and meiotic crossovers (Bowring *et al.* 2006; Celerin *et al.* 2000; Cha *et al.* 2000; Storlazzi *et al.* 2003). In worms and flies, SPO11 is also required for meiotic crossover but not for synapsis (Dernburg *et al.* 1998; McKim and Hayashi-Hagihara 1998). In mammals, SPO11 is required for synapsis and for meiotic sex chromosome inactivation (MSCI) (Bellani *et al.* 2005; Romanienko and Camerini-Otero 2000).

We hypothesize that if PDSBs are required for efficient meiotic silencing then, in the absence of SPO11, we should observe a significant reduction in silencing. To test this, we first compared meiotic silencing of *Rsp* in crosses homozygous for *spo11* relative to heterozygous crosses (Table 2; crosses 1-4). Importantly, silencing was efficient in crosses with or without *spo11*, as demonstrated by production of round-shaped ascospores (Figure 11A and Figure 11C, lower panel). We note that, although many fewer ascospores were observed in the homozygous cross, examination of numerous perithecia of the same cross and

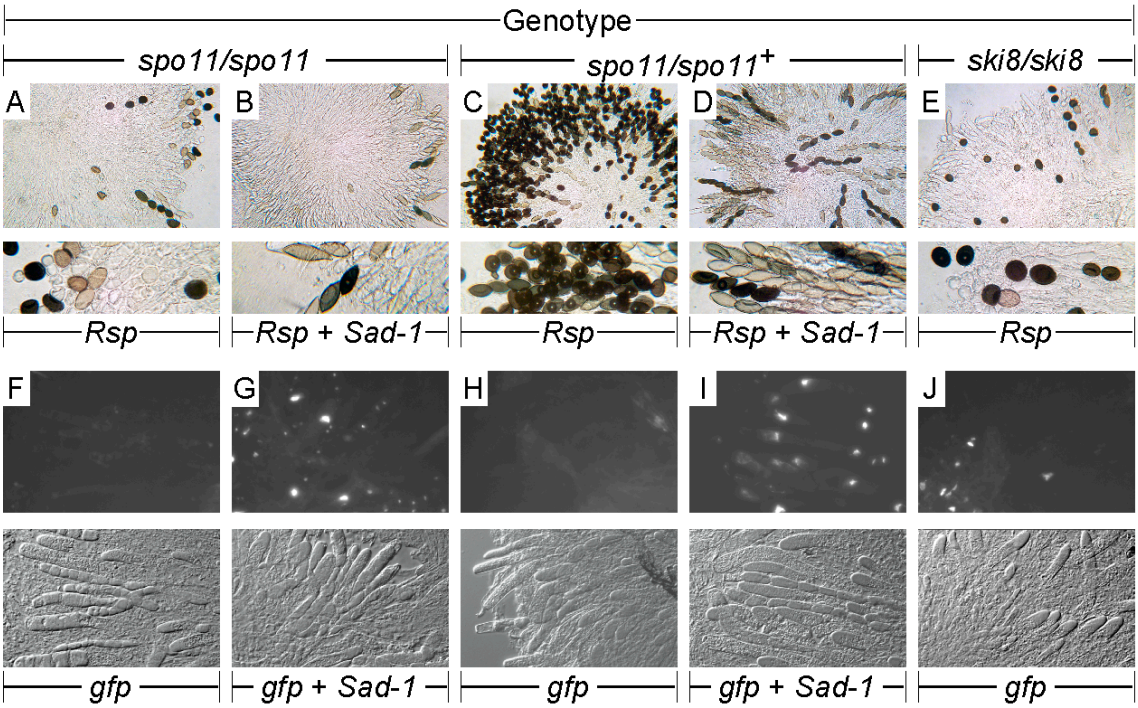

Figure 11. *Spo11*- and *ski8*-dependent programmed double-strand breaks are not required for meiotic silencing. Representative images of selected crosses from Table 2 are shown. Each cross (A to J) is represented by two images. For A to E, the upper images correspond to single rosettes and illustrate the degree of meiotic dysfunction in the mutants, whereas the smaller lower images are a region from the same rosettes but at a higher magnification to accentuate ascospore morphology. For F to J, the upper images report GFP fluorescence, whereas the lower images correspond to Nomarski pictures of the same field. The genotypes of these representative crosses are: A, B, F and G (*spo11* homozygous); C, D, H and I (*spo11* heterozygous); and E and J (*ski8* homozygous). The identity of the "unpaired" reporter genes used in each cross is indicated at the bottom of each image pair.

that of crosses performed at different times revealed that most ascospores were round (Table 2). Furthermore, the formation of round ascospores was dependent on the activity of the *Suppressor of ascus-dominance-1* (*Sad-1*) (Shiu and Metzenberg 2002) (Figure 11B and Figure 11D). Since the suppression of meiotic silencing by *Sad-1*$^{\Delta}$ likely involves the meiotic silencing of *sad-1*$^{+}$, it appears that *spo11* was not required for silencing at this locus either. The pairing-dependence of *Sad-1* is well documented (Pratt *et al.* 2004; Shiu and Metzenberg 2002).

We constructed strains carrying mutant versions of the Neurospora ortholog of the yeast *superkiller8* (*ski8*) gene. *Ski8* was an attractive target for several reasons. 1) In *Sordaria macrospora*, a filamentous fungus closely-related to Neurospora, Ski8 localizes with Spo11 to meiotic chromosomes, where both are required for PDSB formation (Storlazzi *et al.* 2003; Tesse *et al.* 2003). 2) In Drosophila, exosome-mediated decay of 5' fragments generated by RISC cleavage during RNAi requires Ski8 (Orban and Izaurralde 2005). 3) Finally, in yeast, Ski8 protein has a role in controlling dsRNA viruses as part of a heterotrimeric helicase complex involved in mRNA decay (Masison *et al.* 1995). With phenotypes indistinguishable from the *spo11* crosses, *ski8* homozygous crosses were meiotically aberrant and silenced *Rsp* efficiently (Figure 11E; Table 2, crosses 5-8).

By assaying meiotic silencing by ascospore morphology, we were looking at a relatively late phenotype and thus were only able to score meiotic silencing among the asci that had developed to this stage. Therefore, it seemed possible that we might have missed a meiotic silencing defect of these mutants earlier in meiosis. To test this, we used a *hH1*::*gfp* reporter gene driven by the *clock controlled gene-1* (*ccg-1*) promoter that was inserted at the *pan-2* locus on LG VI. The complete experiment with controls and quantification is given in Table 2,

Table 2. Testing the connection between meiotic silencing and meiosis

Cross Number	Selected Crosses	Female[a, b]	Male[b]	Meiotic Mutation(s)[b]	Reporter Gene(s) Unpaired	Predominant Reporter Output (% Wild-type ± SE)[c]	Predominant Meiotic Development[c]
01	11A	RPNCR295A + H	RPNCR329A	*spo11* X *spo11*	*rsp*$^+$	Round spores (0.7±0.3%)	Abnormal
02	11B	RPNCR295A + H	RPNCR305A	*spo11* X *spo11*	*sad-1*$^+$, *rsp*$^+$	Spindle spores (99.4±0.4%)	Abnormal
03	11C	RPNCR295A + H	RPNCR68A	*spo11* X *spo11*$^+$	*rsp*$^+$	Round spores (8.3±3.1%)	Normal
04	11D	RPNCR295A + H	FGSC 8741	*spo11* X *spo11*$^+$	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0%)	Normal
05	11E	RPNCR293A + H	RPNCR330A	*ski8* X *ski8*	*rsp*$^+$	Round spores (2.4±0.4%)	Abnormal
06		RPNCR293A + H	RPNCR317A	*ski8* X *ski8*	*sad-1*$^+$, *rsp*$^+$	Spindle spores (97.9±1.3%)	Abnormal
07		RPNCR293A + H	RPNCR68A	*ski8* X *ski8*$^+$	*rsp*$^+$	Round spores (6.4±2.3%)	Normal
08		RPNCR293A + H	FGSC 8741	*ski8* X *ski8*$^+$	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0%)	Normal
09	11F	RPNCR328A	RPNCT415A	*spo11* X *spo11*	*hH1*$^+$::*gfp*$^+$	GFP Off (2.2±0.4%)	Abnormal
10	11G	RPNCR304A	RPNCT415A	*spo11* X *spo11*	*sad-1*$^+$; *hH1*$^+$::*gfp*$^+$	GFP On (89.5±5.3%)	Abnormal
11	11H	RPNCR331A	RPNCT415A	*spo11* X *spo11*$^+$	*hH1*$^+$::*gfp*$^+$	GFP Off (1.6±0.8%)	Normal
12	11I	FGSC 8740	RPNCT415A	*spo11* X *spo11*$^+$	*sad-1*$^+$; *hH1*$^+$::*gfp*$^+$	GFP On (93.9±1.4%)	Normal
13	11J	RPNCR293A	RPNCT414A	*ski8* X *ski8*	*hH1*$^+$::*gfp*$^+$	GFP Off (5.7±3.0%)	Abnormal
14		RPNCR316A	RPNCT414A	*ski8* X *ski8*	*sad-1*$^+$; *hH1*$^+$::*gfp*$^+$	GFP On (94.3±3.8%)	Abnormal
15		RPNCR331A	RPNCT414A	*ski8* X *ski8*$^+$	*hH1*$^+$::*gfp*$^+$	GFP Off (3.9±3.6%)	Normal
16		FGSC 8740	RPNCT414A	*ski8* X *ski8*$^+$	*sad-1*$^+$; *hH1*$^+$::*gfp*$^+$	GFP On (90.7±2.2%)	Normal

Table 2. Continued

Cross Number	Selected Crosses	Female[a, b]	Male[b]	Meiotic Mutation(s)[b]	Reporter Gene(s) Unpaired	Predominant Reporter Output (% Wild-type ± SE)[c]	Predominant Meiotic Development[c]
17	12A	FGSC 6187	RPNCR430A	*mei-3* X *mei-3*	*hH1*$^+$::*gfp*$^+$	GFP Off (0.0±0.0%)	Abnormal
18	12B	RPNCR482A	RPNCR430A	*mei-3* X *mei-3*	None	GFP On (88.0±3.7%)	Abnormal
19	12C	FGSC 6187	RPNCR417A	*mei-3* X *mei-3*$^+$	*hH1*$^+$::*gfp*$^+$	GFP Off (4.5±3.3%)	Normal
20	12D	RPNCR482A	RPNCR417A	*mei-3* X *mei-3*$^+$	None	GFP On (98.1±1.3%)	Normal
21	13A	RPNCR274A + H	RPNCR174A	*msh4* X *msh4*	*rsp*$^+$	Round spores (0.0±0.0%)	Abnormal
22		RPNCR274A + H	RPNCR182B	*msh4* X *msh4*	*sad-1*$^+$, *rsp*$^+$	Spindle spores (99.5±0.3%)	Abnormal
23		RPNCR274A + H	RPNCR331A	*msh4* X *msh4*$^+$	*rsp*$^+$	Round spores (1.3±0.2%)	Abnormal
24		RPNCR274A + H	FGSC 8740	*msh4* X *msh4*$^+$	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0.0%)	Normal
25	13B	RPNCR272A	RPNCR166A	*mei-1* X *mei-1*	*rsp*$^+$	Round spores (0.0±0.0%)	Abnormal
26		RPNCR272A	RPNCR183A	*mei-1* X *mei-1*	*sad-1*$^+$, *rsp*$^+$	Spindle spores (97.6±0.9%)	Abnormal
27		RPNCR272A	RPNCR331A	*mei-1* X *mei-1*$^+$	*rsp*$^+$	Round spores (1.0±0.2%)	Normal
28		RPNCR272A	FGSC 8740	*mei-1* X *mei-1*$^+$	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0.0%)	Normal
29	13C	RPNCR210A + H	RPNCR168A	*Mei-2* X *Mei-2*	*rsp*$^+$	Round spores (0.4±0.2%)	Abnormal
30		RPNCR210A + H	RPNCR184A	*Mei-2* X *Mei-2*	*sad-1*$^+$, *rsp*$^+$	Spindle spores (98.7±0.9%)	Abnormal
31		RPNCR210A + H	RPNCR211A	*Mei-2* X *Mei-2*	*sms-4*$^+$; *rsp*$^+$	Spindle spores (76.1±6.5%)	Abnormal
32		RPNCR210A + H	RPNCR331A	*Mei-2* X *mei-2*$^+$	*rsp*$^+$	Round spores (5.4±2.1%)	Normal and Abnormal

Table 2. Continued

Cross Number	Selected Crosses	Female[a, b]	Male[b]	Meiotic Mutation(s)[b]	Reporter Gene(s) Unpaired	Predominant Reporter Output (% Wild-type ± SE)[c]	Predominant Meiotic Development[c]
33		RPNCR210A + H	FGSC 8740	*Mei-2* X *mei-2*$^+$	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0.0%)	Normal
34	13D	RPNCR154A + H	RPNCR331A	None	*rsp*$^+$	Round spores (11.5±6.2%)	Normal
35		RPNCR154A + H	FGSC 8740	None	*sad-1*$^+$, *rsp*$^+$	Spindle spores (100.0±0.0%)	Normal
36	13E	RPNCR295A + H	RPNCR327A	*spo11* X *spo11*	None	Round spores (0.1±0.1%)	Abnormal
37		RPNCR293A + H	RPNCR337A	*ski8* X *ski8*	None	Round spores (0.3±0.1%)	Abnormal
38		RPNCR274A + H	RPNCR179A	*msh4* X *msh4*	None	Round spores (0.4±0.3%)	Abnormal
39		RPNCR272A	RPNCR167A	*mei-1* X *mei-1*	None	Round spores (0.1±0.1%)	Abnormal
40		RPNCR210A + H	RPNCR169A	*Mei-2* X *Mei-2*	None	Round spores (0.3±0.2%)	Abnormal
41		RPNCR154A + H	RPNCR334A	None	None	Round spores (0.2±0.1%)	Normal

[a] + H indicates that the female was a heterokaryon with FGSC 4564. Heterokaryons were used to normalize female-sterility defects of mutant strains. Although FGSC 4564 can readily complement any pre-dikaryotic defects, the *mating-type a*^{m1} mutant allele present in this nucleus prevents it from entering ascogenous tissue, thus blocking its participation in meiosis (Perkins, 1984).

[b] See Materials and Methods for details on the construction of strains and Table A1 for complete genotypes and allele information.

[c] "Abnormal" and "Normal" meiotic development indicate that >99% of the asci had an abnormal or normal ascus phenotype during development. However at the time silencing was assayed for *mei-3* mutants, the ascus phenotype was indistinguishable from wild type. Therefore, for *mei-3* meiotic development refers to later time points.

crosses 9-16. *HH1::gfp* silencing was independent of *spo11* (Figure 11, compare F and H) and *ski8* (Figure 11J) but was *Sad-1*-dependent (Figure 11, compare F and G; H and I). In wild type and mutant crosses, GFP signal was regularly detected in the pre-meiotic asci, which were concentrated at the center of the rosette (lower left corner of Figure 11J). Importantly, this signal does not overlap with meiotic asci, and was therefore no included in our quantification of meiotic silencing. asci, it tended to be in the youngest of asci, which may represent the persistence of GFP protein in the presence of silencing rather than the absence of silencing. A *spo76*::*gfp* and *hH1*::*gfp* fusion at their canonical position (below), were also silenced efficiently in *spo11* and *ski8* mutants, respectively (data not shown). Together these results demonstrate that *spo11*- and *ski8*-dependent PDSBs are not required for meiotic silencing.

It is unknown, however, if any other DNA breaks arising by another mechanism act as substrates for homology searching in Neurospora. It is known that, unlike gamma-irradiation induced double-strand breaks, these hypothetical substrates do not contribute to synapsis (Bowring *et al.* 2006). Nonetheless, they could be required for meiotic *trans*-sensing and play an important role in comparing chromosomal regions early in meiosis. In support of an additional recombination pathway, Neurospora *spo11* mutants exhibit normal crossover frequencies in one genetic interval on LG I. Unfortunately, it is unknown if these crossovers occurred in meiosis. Such a DNA-based homology searching mechanism would likely involve strand invasion to allow base-pairing between homologues and, therefore, the activities of a RecA/Rad51 recombinase protein (Baumann and West 1998). MEI-3 is the only Neurospora protein in this enzyme family and it is important for mitotic homologous recombination (Borkovich *et al.* 2004). The chromosome pairing defects and meiotic arrest of *mei-3* mutants further suggest a role for MEI-3 in meiotic recombination (Cheng *et al.* 1993).

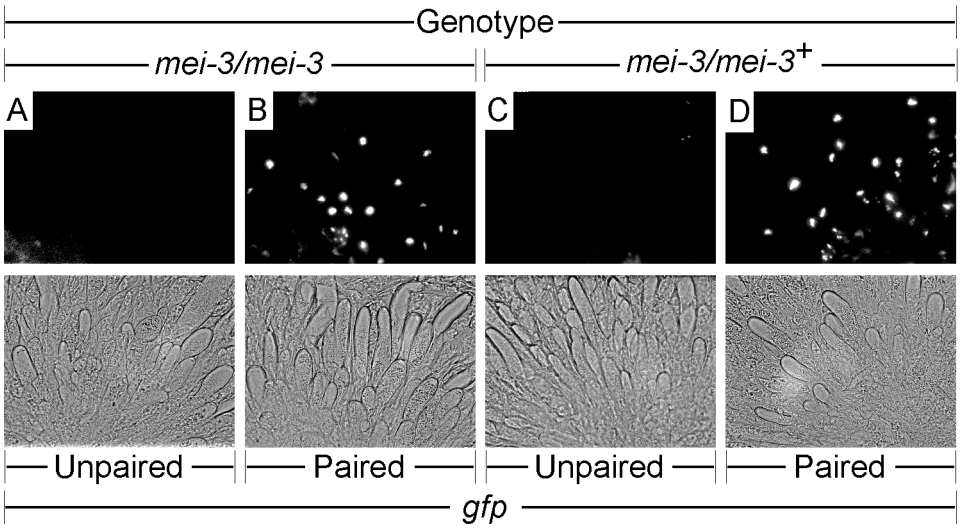

Figure 12. *Mei-3*, the sole RecA/Rad51 recombinase of Neurospora, is not required for meiotic silencing. Representative images of selected crosses from Table 2 are given. Each cross is represented by two images where the upper images report GFP fluorescence (no silencing) or lack of it (silencing) and the lower ones correspond to bright-field images of the same field. The genotypes of these representative crosses are: A and B (*mei-3* homozygous) and C and D (*mei-3* heterozygous). Crosses represented by A and C carry "unpaired" *gfp* DNA, whereas crosses represented by B and D carry "paired" *gfp* DNA. In these crosses "paired" signifies that one parent carried a functional, and the other carried a non-functional *gfp* allele (a frameshift allele) at the *histone-H1* chromosomal position. Note that “paired” and “unpaired” refers solely to the presence or absence of *gfp* DNA at allelic positions in the parents and does not refer to any specific form of homologue pairing.

To test the existence of such a hypothetical mechanism, we assayed the silencing of the *hH1*::*gfp* reporter driven by its own promoter at its canonical location on LG VII in *mei-3* mutants (Table 2, crosses 17-20). Silencing of the unpaired *gfp* reporter gene was proficient in both homozygous (Figure 12A) and heterozygous (Figure 12C) *mei-3* crosses. In the control paired condition, both homozygous (Figure 12B) and heterozygous (Figure 12D) crosses for *mei-3* showed no silencing.

Without a requirement for the primary recombinase or proteins required for the formation of PDSBs, it becomes difficult to imagine that *trans*-sensing in Neurospora occurs through the established meiotic recombination pathways.

Synapsis, meiotic crossovers and proper chromosome segregation are also dispensable for meiotic silencing

Given that meiotic silencing in mouse and *C. elegans* is directly correlated with the inability of the chromatin on unpaired DNA regions to synapse (Baarends *et al.* 2005; Bean *et al.* 2004; Turner *et al.* 2005), and that Neurospora also silences unpaired DNA regions (Aramayo and Metzenberg 1996; Shiu *et al.* 2001), it is reasonable to propose a similar relationship in Neurospora. One could argue that *trans*-sensing in Neurospora requires DNA pairing, and since synapsis is chromatin based and DNA sequence-independent, that synapsis should not be the mechanism guiding silencing. However, in our opinion, the sequence-dependence is an overstated assumption of Neurospora silencing that lacks direct evidence and is based solely on the behavior of RIP alleles whose allelic differences with their pairing partner are multilayered (see Chapter II).

We therefore tested the involvement of synapsis in meiotic *trans*-sensing by determining the extent of meiotic silencing in *msh4*, *mei-1* and *Mei-2* mutants

(Table 2, crosses 21-35). *Msh4* has not yet been characterized fully in Neurospora, but shows clear sporulation defects (Conway *et al.* 2006). It was selected due to its early role in designating interference-associated crossovers, which are proposed to immediately precede synapsis and contribute to its initiation (Borner *et al.* 2004; Fung *et al.* 2004). *Mei-1* and *Mei-2* gene products have not been identified; however their mutant phenotypes have been well characterized. Both mutants are defective in stable homologue synapsis and meiotic crossover (Lu and Galeazzi 1978; Schroeder and Raju 1991; Smith 1975).

Aside from the meiotic defects observed in these mutants, silencing of *Rsp* was efficient in homozygous *msh4* (Figure 13A), *mei-1* (Figure 13B), and *Mei-2* (Figure 13C) crosses (Table 2, crosses 21-35). The round ascospores produced in these crosses were indistinguishable from the spores produced in wild-type crosses containing only one unpaired copy of *Rsp* (Figure 13D), or by homozygous control crosses carrying no functional copies of *Rsp* (Figure 13E, crosses 36-41).

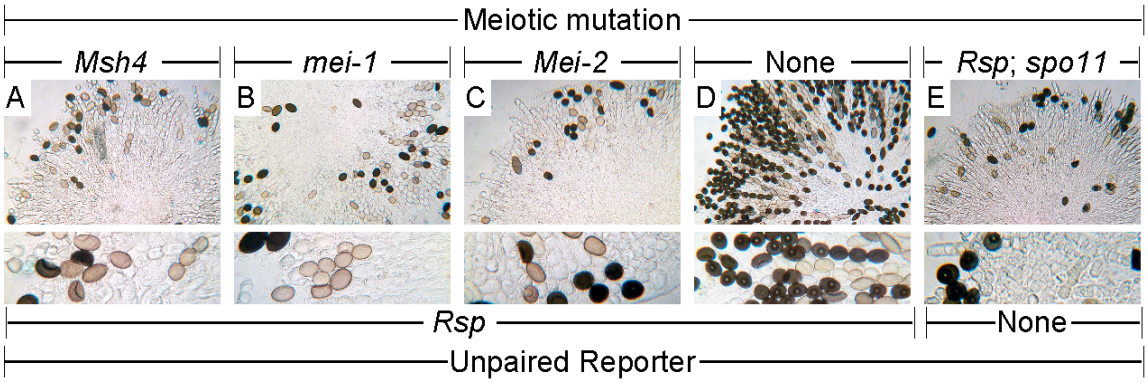

Figure 13. Synapsis, meiotic crossovers and proper chromosome segregation are dispensable for meiotic silencing. Representative images of selected crosses from Table 2 are shown. As for Figure 11 A-J, each cross is represented by two images, where the upper images report single rosettes, and the smaller lower images are from the same rosettes but at a higher magnification. The meiotic marker tested in the homozygous condition is indicated at the top of each picture, whereas the presence or absence of "unpaired" *Rsp* DNA in each cross is indicated at the bottom of the figure.

The timing of arrest and the dependency on PDSBs distinguishes the developmental arrest of *Sad-1* from recombination genes

One of the most amazing and poorly understood phenotypes observed among the known suppressors of meiotic silencing is the meiotic block of *Sad-1* mutants. Crosses homozygous for *Sad-1* arrest in prophase I at pachytene or diplotene (Figure 14A; (Shiu *et al.* 2001). Additionally, crosses homozygous for *Sad-2,* a gene required for proper SAD-1 perinuclear localization, have similar meiotic defects and additionally present partial abnormalities in chromosome pairing (Shiu *et al.* 2006).

Why would an RNA-dependent RNA polymerase be required for meiosis? We hypothesized that *Sad-1* mutants are blocked in meiosis because they are unable to resolve recombination intermediates and therefore activate pachytene checkpoint (PCH). Similarly, many meiotic mutants failure to resolve recombination intermediates resulting in PCH activation and meiotic arrest (Roeder and Bailis 2000). A pachytene checkpoint has not been formally established in Neurospora. However, mutants of Neurospora orthologs of recombination proteins that trigger PCH when mutant in yeast also arrest in prophase I in Neurospora (Raju and Perkins 1978).

It is well established that PCH can be partially bypassed when arresting mutants are combined with *Spo11* loss-of-function alleles (Bellani *et al.* 2005; Leu *et al.* 1998; Storlazzi *et al.* 2003). In this situation, no recombination intermediates form to trigger PCH due to the lack of PDSBs (Roeder and Bailis 2000). Therefore, we used genetic crosses to test first if PDSB mutants could suppress the meiotic arrest of *mei-3* and *mutagen sensitive-21* (*mus-21*), the Neurospora ortholog of mammalian DNA damage response protein ATM (Borkovich *et al.* 2004). As expected, when combined with *spo11* or *ski8*, the barren phenotype of

Sad-1 mutants was partially suppressed (Figure 14E and Figure 14F, respectively; Table 3, crosses 50-59). Following a similar logic, we constructed strains containing *Sad-1* and either *spo11* or *ski8* and tested their meiotic behavior. We did not observe suppression of the *Sad-1* meiotic defect by either *spo11* (Figure 14B) or *ski8* (Figure 14C, Table 3, crosses 42-43). These results suggest that the meiotic arrest observed in *Sad-1* mutants is unrelated to the same meiotic recombinational block as *mei-3* and *mus-21*.

Finally, two other genes struck our interest as candidates for meiotic *trans*-sensing and meiotic silencing. *Mus-9* is the ortholog of mammalian ATR, which is known to associate with unpaired chromatin in mouse meiosis (Turner *et al.* 2005) and is associated with DNA damage response, particularly in S-phase in response to ssDNA bound by single stranded-binding protein RPA (Bartek *et al.* 2004; Dart *et al.* 2004; Zou and Elledge 2003). Another recombinational protein Rad52, *mus-11* in Neurospora, also interacts with RPA (Sugiyama and Kowalczykowski 2002). Indeed both Rad52 and ATR co-localize with hyperphosphorylated RPA in response to DNA damage (Wu *et al.* 2005). If *trans*-sensing occurs through single stranded DNA gaps as opposed to PDSBs, one could hypothesize a role for these proteins in *trans*-sensing. However, close inspection of the development in crosses homozygous for these mutants revealed an arrest very soon following fertilization, perhaps even before karyogamy, as evidenced by the absence of meiotic asci (Table 3, crosses 60 and 63). MRN complex mutants in Neurospora also arrest around this stage in development (Raju and Perkins 1978), and in Arabidopsis an MRN mutant results in Spo11-dependent genome fragmentation in meiosis (Puizina *et al.* 2004). We could hypothesize that *spo11*-dependent genome fragmentation occurs in these early arrest mutants of Neurospora. However, consistent with a pre-meiotic arrest, yet contrary to *mei-3*, *mus-21* and the MRN mutants of Arabidopsis, the barren phenotype of these mutants was not suppressed by

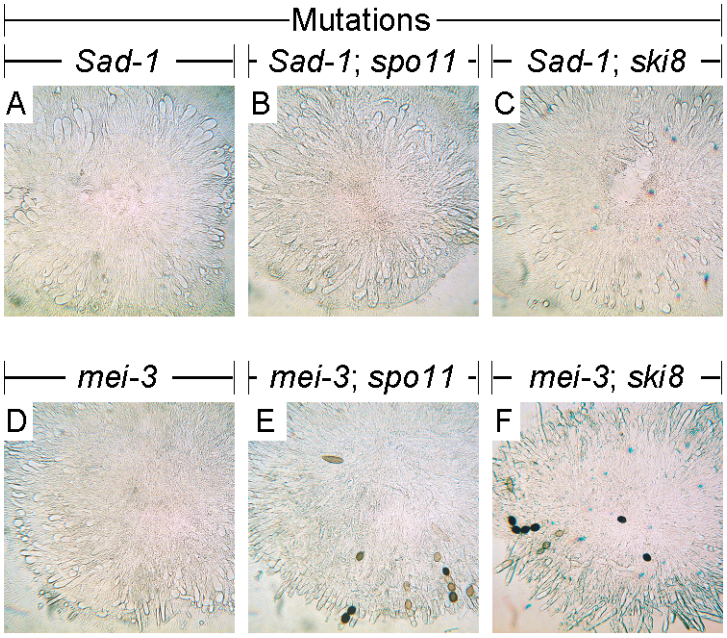

Figure 14. The meiotic arrest of *mei-3*, but not *Sad-1*, is suppressed by mutants in PDSB. Representative images from selected crosses described in Table 3 are presented. The mutant loci tested in the homozygous condition are indicated above each image. Note that the crosses represented in D, E and F additionally contained an "unpaired" copy of *Rsp* which was silenced.

Table 3. Testing the requirements of PDSBs for developmental arrest

Cross Number	Selected Crosses	Female[a, b]	Male[b]	Meiotic Mutation(s)[b]	Spores Formed?[c]	Meiotic Development?[d]
42	14A	RPNCR304A + H	FGSC 8741	*Sad-1*; *spo11* X *Sad-1*; *spo11*$^+$	No	Abnormal
43	14B	RPNCR304A + H	RPNCR305A	*Sad-1*; *spo11* X *Sad-1*; *spo11*	No	Abnormal
44		RPNCR304A + H	RPNCR329A	*Sad-1*; *spo11* X *sad-1*$^+$; *spo11*	Yes	Abnormal
45		RPNCR304A + H	RPNCR68A	*Sad-1*; *spo11* X *sad-1*$^+$; *spo11*$^+$	Yes	Normal
46		RPNCR316A + H	FGSC 8741	*Sad-1*; *ski8* X *Sad-1*; *ski8*$^+$	No	Abnormal
47	14C	RPNCR316A + H	RPNCR317A	*Sad-1*; *ski8* X *Sad-1*; *ski8*	No	Abnormal
48		RPNCR316A + H	RPNCR330A	*Sad-1*; *ski8* X *sad-1*$^+$; *ski8*	Yes	Abnormal
49		RPNCR316A + H	RPNCR68A	*Sad-1*; *ski8* X *sad-1*$^+$; *ski8*$^+$	Yes	Normal
50	14D	RPNCR307B + H	FGSC 6187	*mei-3*; *spo11* X *mei-3*; *spo11*$^+$	No	Abnormal
51	14E	RPNCR307B + H	RPNCR306A	*mei-3*; *spo11* X *mei-3*; *spo11*	Yes – Round	Abnormal
52		RPNCR307B + H	RPNCR328A	*mei-3*; *spo11* X *mei-3*$^+$; *spo11*	Yes – Round	Abnormal
53		RPNCR307B + H	RPNCR331A	*mei-3*; *spo11* X *mei-3*$^+$; *spo11*$^+$	Yes – Round	Normal
54		RPNCR319A + H	FGSC 6187	*mei-3*; *ski8* X *mei-3*; *ski8*$^+$	No	Abnormal
55	14F	RPNCR319A + H	RPNCR318A	*mei-3*; *ski8* X *mei-3*; *ski8*	Yes – Round	Abnormal
56		RPNCR319A + H	RPNCR331A	*mei-3*; *ski8* X *mei-3*$^+$; *ski8*$^+$	Yes – Round	Normal and Abnormal
57		RPNCR383A	RPNCR375A	*mus-21*; *spo11* X *mus-21*; *spo11*$^+$	Rare	Abnormal

Table 3. Continued

Cross Number	Selected Crosses	Female[a, b]	Male[b]	Meiotic Mutation(s)[b]	Spores Formed?[c]	Meiotic Development?[d]
58		RPNCR383A	RPNCR384A	*mus-21*; *spo11* X *mus-21*; *spo11*	Yes	Abnormal
59		RPNCR383A	RPNCR329A	*mus-21*; *spo11* X *mus-21*$^{+}$; *spo11*	Yes	Abnormal
60		RPNCR387A	RPNCR392A	*mus-9*; *spo11* X *mus-9*; *spo11*$^{+}$	No	None
61		RPNCR387A	RPNCR388A	*mus-9*; *spo11* X *mus-9*; *spo11*	No	None
62		RPNCR387A	RPNCR329A	*mus-9*; *spo11* X *mus-9*$^{+}$; *spo11*	No	None
63		RPNCR385A	RPNCR376A	*mus-11*; *spo11* X *mus-11*; *spo11*$^{+}$	No	None
64		RPNCR385A	RPNCR386A	*mus-11*; *spo11* X *mus-11*; *spo11*	No	None
65		RPNCR385A	RPNCR329A	*mus-11*; *spo11* X *mus-11*$^{+}$; *spo11*	No	None

+H indicates that the female was a heterokaryon with FGSC 4564. Heterokaryons were used to normalize female-sterility defects of mutant strains. Although FGSC 4564 can readily complement any pre-dikaryotic defects, the *mating-type a*^{m1} mutant allele present in this nucleus prevents it from entering ascogenous tissue, thus blocking its participation in meiosis (Perkins, 1984).

[b]See Materials and Methods for details on the construction of strains and Table A1 for complete genotypes and allele information.

[c]"Yes" and "No", indicate respectively that either ascospores were observed or no mature ascospores were observed. Feeble white ascospores can be difficult to discern and rare ones could have been missed. "Rare" for cross #57 is to indicate that frequently rosettes contained one or two asci with ascospores and these most frequently contained eight ascospores; importantly, however, this was much lower than the number of ascospore-containing asci present in cross #58. *Rsp* was unpaired in crosses 50 – 56. "Round" for these crosses indicates that the vast majority of spores produced had a round morphology.

[d]"Abnormal" and "Normal" meiotic development indicate that >90% of the asci had an abnormal or normal meiotic phenotype at some time in development. For cross #48, the presence of both normal and abnormal meiotic development seems to be due to a synthetic effect of the mutations. None indicates that no asci/meiotic cells were observed.

spo11 (Table 3, crosses 60-65). Unfortunately, the developmental arrest of *mus-9* and *mus-11* mutants has prevented us from assaying meiotic silencing in the complete absence of these gene products.

DISCUSSION

Meiotic *trans*-sensing and chromosome pairing

In the context of meiotic silencing in Neurospora, pairing must occur between allelic regions on homologous chromosomes, i.e., merely having two copies of a gene in the diploid genome is insufficient to satisfy *trans*-sensing (Aramayo and Metzenberg 1996). The simplest hypothesis would postulate that *trans*-sensing be mediated through known homologous chromosome pairing processes. However, mutants in components typically required for these processes still exhibited efficient meiotic silencing. Unfortunately, Neurospora lags significantly behind other model organisms in the characterization of the roles of recombination in meiotic pairing. Thus, our conclusions are limited to the extent to which the mutant phenotypes are established in Neurospora and the extent to which one is willing to extrapolate to Neurospora the role of these genes in better-characterized systems.

Given what is known of the mutants we have tested in Neurospora, synapsis is not required for meiotic silencing. Locus-specific silencing was observed in genomes that globally lacked synapsis. This is in contrast to the silencing of unpaired chromatin observed in mouse and *C. elegans*, where there is a strict relationship between silencing and synapsis (Baarends *et al.* 2005; Turner *et al.* 2005).

Meiotic recombination cannot be definitively measured genetically in the mutants tested. Some mutants failed to produce progeny from which to score recombination (e.g., *mei-3*), and others produced aneuploidy progeny that can

undergo somatic recombination between homologous chromosomes (e.g., *spo11*). However, the role of PDSBs and the RecA recombinase in recombination is well established (reviewed Krogh and Symington 2004; Zickler and Kleckner 1999). In the closely related filamentous fungus *Sordaria macrospora*, application of microscopy techniques has shown that recombination is greatly dependent on PDSBs (Storlazzi *et al.* 2003; Tesse *et al.* 2003). In *spo11* mutants, only rare nuclei contain even a single Rad51 focus, presumably the site of recombination, compared to ~50 foci in wild-type nuclei. In addition, chiasmata are virtually absent at diplotene (Storlazzi *et al.* 2003).

Measurement of long-distance chromosome pairing, or chromosome alignment, also requires challenging microscopy techniques (Gerton and Hawley 2005). For this reason, it has only been well characterized globally in one organism that has a synaptic meiosis, *S. macrospora.* In Sordaria it is known that stable chromosome interaction at any level is fully dependent on the presence of *spo11*- and *ski8*-derived PDSBs. These proteins *per se* are not required however; rather the presence of recombinogenic double-strand breaks are needed since the level gamma-irradiation induced double-strand breaks in the absence of these proteins correlates well with the level of pairing between homologous chromosomes (Storlazzi *et al.* 2003; Tesse *et al.* 2003; Zickler 2006).

How similar are *S. macrospora* and *N. crassa*? Sordaria and Neurospora are in the same taxonomic family and share very similar development following fertilization, even sharing similar ascus dimensions (Carr and Olive 1958; Raju 1980). At the molecular level, they are estimated to have diverged from their last common ancestor less than 36 MYA, their exons are on average 90% identical at the DNA level, and they have the same number of chromosomes (Wu *et al.* 1998; Nowrousian *et al.* 2004; Carr and Olive 1958). Importantly, it is

not yet known if Sordaria exhibit significant meiotic silencing. However, some evidence suggests it might; heterozygosity for a karyotypic polymorphism correlates with a dominant ascus development defect as would be expected for meiotic silencing by unpaired DNA (Poggeler *et al.* 2000).

With the currently available information, as far as the role of recombination and chromosome alignment in meiotic silencing go, it seems to boil down to one question: what is more likely, stable chromosome pairing- and recombination-independent meiotic *trans*-sensing or the significant divergence of the meiotic recombination pathway of *Neurospora crassa* and *Sordaria macrospora*? Either way, clearly two interesting possibilities remain: either Neurospora has a novel recombination pathway or it has a recombination-independent mechanism for intimate homologue comparison.

Is there chromosome comparison in the absence of PDSBs? Several pieces of evidence suggest an intrinsic ability of homologues to unstably pair in the absence of PDSBs (Gerton and Hawley 2005). The mechanisms governing the homology recognition of these interactions are unknown. A recent thermodynamic model has been proposed whereby unknown sequence-specific DNA-binding proteins could theoretically drive chromosome association in a concentration-dependent manner (Nicodemi *et al.* 2008). In *S. cerevisiae*, FISH analysis has shown that homologous associations in *Spo11* mutants are reduced relative to wild type but are nonetheless more prevalent then heterologous associations (Loidl *et al.* 1994; Weiner and Kleckner 1994). Also, centromere and telomere associations contribute to homologue alignment, but the stability of the alignment is still *Spo11*-dependent (Peoples-Holst and Burgess 2005; Tsubouchi and Roeder 2005). Therefore, we could postulate that sufficient homologue comparison occurs through these unstable interactions to allow for meiotic *trans*-sensing and the triggering of

meiotic silencing. Alternatively, the events governing meiotic *trans*-sensing may be mechanistically unrelated to homologous chromosome pairing (see Chapter V).

How are homologous chromosomal regions compared in meiosis?

We present the following models to compare chromosome pairing and meiotic silencing in Neurospora and mouse. These differ primarily on when unpaired DNA is detected and on the dependence of double-stranded breaks for its detection. At the beginning of meiosis, unknown molecular machineries (Figure 15, "Black Box") direct *trans*-sensing and unstable homologue pairing between paternal and maternal homologues. At this point DNA is scored as "paired" or "unpaired". In Neurospora, DNA found to be "unpaired" is silenced throughout the course of meiosis without further evaluation (Arrow 1). Regardless of the paired condition, Spo11 will introduce double-strand breaks (Mahadevaiah *et al.* 2001). In the absence of a homologous region or chromosome to repair or synapse with, a pachytene checkpoint could be activated in mouse. These unrepaired PDSBs could be the substrates for a BRCA1- and ATR-dependent second wave of gamma-H2AX formation, which would be followed by meiotic silencing and sex-body formation (Arrow 2) (Bellani *et al.* 2005; Fernandez-Capetillo *et al.* 2003; Turner *et al.* 2004; Turner *et al.* 2005). In Neurospora, the role of ATR and gamma-H2AX in meiotic silencing is unknown. However, if ATR were to respond to a substrate other than PDSBs, as postulated for mouse (below), it is conceivable that meiotic silencing in Neurospora is ATR-dependent.

In paired regions, PDSBs are the substrate for ATM-dependent gamma-H2AX formation and the initiation of RecA-mediated recombination, which results in stable homologue juxtaposition and alignment. This is followed by crossover interference, synapsis and the repair of PDSBs. Regardless of the relevance of PDSBs for meiotic silencing, paired regions would not be silenced (Arrow 3).

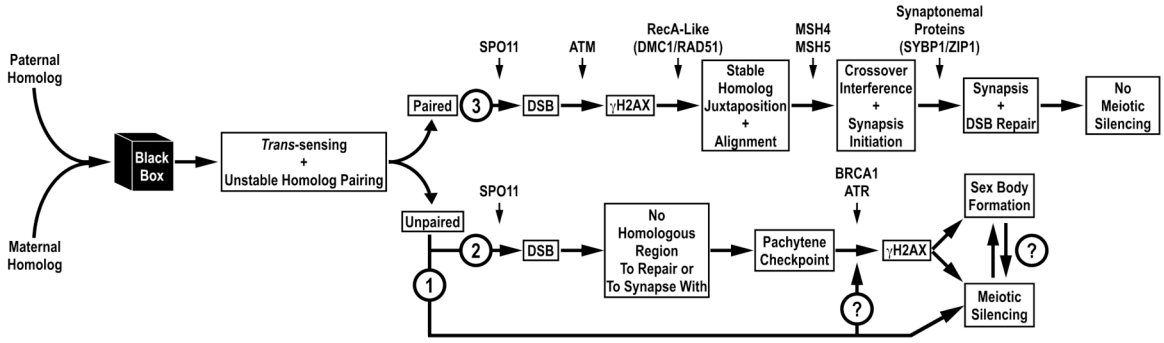

Figure 15. How are homologous chromosomal regions compared in meiosis? Diagrammed is a model for the pathways for homologue pairing and the detection of unpaired DNA. Key steps in the pathways are indicated in boxes. Genes important for progression between steps that have been either tested in this work or mentioned in the discussion are indicated above the arrows between key steps. In Neurospora, it seems that unpaired DNA must be identified early in meiosis independent of DSBs (Arrow 1), but it is not yet known if ATR-dependent H2AX phosphorylation is involved. In mouse, given the roles of BRAC1 and ATR in DNA damage response and the correlation between synapsis and silencing, a simple model for meiotic silencing is presented by Arrow 2. However, we note that depending on the relationship between meiotic silencing (mechanism of gene silencing) and sex-body formation (mechanism of chromosome condensation and compartmentalization), meiotic silencing in mouse could also be DSB-independent (Arrow 1) although certainly dependent upon ATR. In the absence of unpaired DNA, no meiotic silencing is observed (Arrow 3). See discussion for details.

Given the role of BRAC1 and ATR in DNA damage response (Richardson *et al.* 2004), this is the simplest model for mouse. It lacks, however, a demonstration that unrepaired PDSBs are indeed the substrates responded to by BRAC1 and ATR. Therefore, it remains possible that, as we proposed for Neurospora, the determination of unpaired versus paired DNA occurs earlier through a related *trans*-sensing mechanism. Unpaired DNA detected by this *trans*-sensing could be directly acted upon by BRAC1 and ATR, independent of PDSBs, to cause meiotic silencing. This model predicts that, in spermatogenesis of *Spo11, Msh5, and Sypb1* mutants, silencing would still be limited to the unpaired regions of X and Y, even though sex-bodies do not form (Bellani *et al.* 2005; de Vries *et al.* 2005; Mahadevaiah *et al.* 2001). A further prediction would be that the global silencing signatures seen in *Sypb1* and *Msh5* mutants are not actually sites of transcriptional repression (de Vries *et al.* 2005; Mahadevaiah *et al.* 2001). This latter prediction is more difficult to test and assumes that there is sufficient machinery for effective global silencing. The expression of Y-encoded genes early in mouse spermatogenesis suggests that if this early *trans*-sensing is used in mouse, the unpaired DNA that is identified is not acted upon by meiotic silencing until late in pachytene (Wang *et al.* 2005). Although this chromosomal *trans*-sensing may not be the mechanism that triggers meiotic silencing in mouse, it still may be a fundamental mechanism of early chromosome pairing in meiosis.

Conclusion

This initial investigation into the interconnection of meiotic chromosome biology and meiotic *trans*-sensing and silencing has shown that a simple relationship between recombination and synapsis with meiotic silencing most likely does not exist. Alternatively, Neurospora has developed a novel recombination pathway that does not utilize the gene products required for most meiotic recombination. Despite decades of research into DNA and chromosome pairing, the mechanism

of sensing that occurs in Neurospora meiosis remains elusive but all more fascinating. This work thus opens the door for testing hypothesis about homology-sensing mechanisms that previously seemed unlikely. Consequently, we hope that our ongoing characterization of mutants defective in meiotic *trans*-sensing and silencing will lead to a greater understanding of how chromosomes sense each other and how unpaired DNA is both detected and silenced in meiosis. This knowledge is essential to understand the processes required for normal human fertility.

MATERIALS AND METHODS

Molecular biology

Procedures for cloning, DNA analysis, sequencing, Southern blot analysis, and other nucleic acid manipulations were performed as described (Pratt and Aramayo 2002, Pratt, 2004 #454).

Strain description and manipulation

All *N. crassa* strains used in this study are described in Table A1. The formulas for the Vogel's Medium N, the Westergaard's Medium, and the sugar mixture of Brockman and de Serres have been described by Davis and de Serres (1970). *Escherichia coli* K12 XL1-Blue MR (Stratagene) was the host for all bacterial manipulations. Similarly, growth conditions, conidial spheroplast preparation and fungal transformation were performed as described (Pratt and Aramayo 2002). Homokaryon purification was performed as described (Lee *et al.* 2003a; Pratt and Aramayo 2002).

Construction of alleles and mutant strains

Msh4: A 3.0-kbp region of the *msh4* (NCU10895.2) locus was amplified by PCR using oligonucleotides ORP134 and ORP135 from the Sachs/Orbach cosmid G8:A7 (this and other cosmids obtained were from the FGSC). The *Spe*I-

digested product was inserted into the *Xba*I site of the *pan-2* integration plasmid pRATT42b, yielding pRATT72. Following insertion at *pan-2*, and homokaryon purification, the duplication-carrying strain, was crossed to RPNCR62A to invoke RIP-mutagenesis. A selected duplication-containing progeny was then crossed to RPNCR103A to further RIP-mutagenize the *msh4* locus. Among these progeny, RIP-alleles were screened by Southern blot of *Bam*HI-digested genomic DNA. We observed that the isolate RPNCR174 contains, in addition to an *msh4*^{RIP} allele, a spontaneous, unlinked, morphological mutation we called *kinky*. Inactivation of the *msh4* gene was verified by amplification of the RIP allele by PCR using oligonucleotides ORP225 and ORP226 and then sequenced. The sequence was compared using the recent manual annotation (Conway *et al.* 2006) (Figure 10C). The true gene structure has not been experimentally determined. Regardless, the most conserved region of the protein was obliterated by RIP and the allele was heavily methylated and was thus likely transcriptionally repressed (Rountree and Selker 1997).

Ski8: A 1.9-kbp *Eco*RI-*Xba*I fragment containing the *ski8* (NCU03517.2) gene was isolated from the Sachs/Orbach cosmid G3:D6 and subcloned into the same restriction sites of the *pan-2* insertion vector pRATT42b, yielding pRATT105. A homokaryotic *pan-2* insertion-carrying strain derived from RPNCR38A was crossed to RPNCR275A to induce RIP in the absence of meiotic silencing. Two RIP-alleles were identified by Southern blot and selected among progeny lacking the duplication. Inactivation of the gene was verified by amplification of the RIP-alleles by PCR with oligonucleotides ORP183 and ORP184 and sequenced (Figure 10B).

Spo11: A 1.2-kbp EcoRI-XbaI fragment containing the *spo11* (NCU01120.3) gene was isolated from the Sachs/Orbach cosmid G12:F10 and sub-cloned into these sites of the *pan-2* insertion vector pRATT42b, yielding pRATT106. The

locus was subjected to RIP as described for *ski8*. The RIP alleles were amplified by PCR with oligonucleotides ORP179 and ORP180 and sequenced (Figure 10A). Wild-type sequence used for comparison is from Bowring et al. (2006).

Mei-3: We used allele CF-3 from the FGSC collection. To determine the molecular nature of this mutant we amplified the locus from the genomes of strains FGSC 6187 and FGSC 6188 by PCR with oligonucleotides ORP208 and ORP209 and sequenced the amplified product. The sole mutation is a nonsense mutation that changes the cystine-325 codon 5'-TGT-3' to the stop codon 5'-TAA-3'. While the mutation leaves most of the protein intact, the conserved recombinase domain is truncated.

hH1^{+}::*gfp*$^{+}$ alleles: At *pan-2* locus: For completely unrelated purposes the *Bam*HI-*Bgl*II fragment containing the *inl*$^{13\text{-}8}$ allele was sub-cloned from pRATT13m13-8 (Pratt and Aramayo 2002) into the *Bam*HI site of the *pan-2* insertion vector pRATT42b such that *pan-2* and *inl* transcription are in the same direction yielding plasmid pRATT58a. We took advantage of the restriction sites in this recombinant plasmid to replace the entire *inl* insert from *Sac*I to *Not*I with the 3.5-kbp *Sac*I-*Not*I fragment from pMF280 (Folco *et al.* 2003) containing the *hH1*$^{+}$::*gfp*+ fusion driven by the *ccg-1* promoter yielding plasmid pRATT116.

hH1 locus: We had significant difficulty obtaining viable ascospores containing the *ccg-1*(p)::*hH1*$^{+}$::*gfp*$^{+}$ fusion when constructs were inserted at *his-3* or *pan-2* loci in a number of different strains in the absence or presence of RIP (data not shown). To overcome this problem we fused *gfp* to the C-terminus of *hH1* at its canonical *hH1* locus by homologous recombination. The targeting vector, pRATT120, was the product of a three-fragment-ligation between the 3.3-kbp *Nsi*I-*Hin*dIII fragment from pRATT18c (to be described elsewhere), the 2.2-kbp *Pst*I-*Bam*HI fragment from pMF280 and the 0.8-kbp *Hin*dIII-*Bam*HI fragment

obtained by PCR amplification with oligonucleotides ORP202 and ORP203. The expression of *hH1*$^{+}$::*gfp*$^{+}$ fusion in perithecial maternal tissue increases background signal making visualization of GFP in asci difficult. To overcome this problem, we constructed a loss-of-function allele of *hH1*$^{+}$::*gfp*$^{+}$, capable of pairing with its wild-type counterpart. For this, a frameshift and stop mutation were both introduced in the linker region between *hH1*$^{+}$ and *gfp*$^{+}$ by filling-in the *Pac*I site between the two genes yielding pRATT120*. In theory, the *hH1* should be functional, but this was not tested. pRATT120 and pRATT120* were used to transform *mus-51* mutant strains due to their reduced rates of non-homologous recombination (Ninomiya *et al.* 2004).

Neurospora genetics

Non-directional crosses were performed as described previously (Pratt and Aramayo 2002). For directional crosses, strains were all grown in 1 ml of supplemented Vogel's media prior to setting the crosses. In this case, the female was point-inoculated onto Westergaard's solid crossing media in Petri dishes and then incubated at 24°C to 26ºC for 5 to 8 days. Conidia of the males, suspended in media, were then spotted onto different regions of the same female mycelium, using a cotton swab. Crosses were incubated at 24°C to 26ºC. For each cross, 10 to 20 perithecia were dissected. When assaying for ascospores, perithecia were harvested at 12, 15 and 18 days post-fertilization. When assaying for *gfp* expression, perithecia were harvested at 4, 5 and 6 days post-fertilization.

Preparation of perithecial tissues

To obtain rosettes, perithecia were gently plucked off the solid media and placed in a 200 µl tube containing 100 µl of STP solution (50 mM Tris HCl-pH 7.5, 20% PEG 3355, 20% sorbitol). Individually, perithecia were placed into a small puddle (~10-20 µl) of STC on a glass slide. Under a dissecting microscope, perithecia

were then held firm with forceps and a side of the perithecial wall was chopped off with a razorblade. The rosettes were then teased out by squishing the perithecium with forceps and the perithecial tissue then transferred to a second tube (200 µl) containing 100 µl of STP. All the rosettes for a given cross were placed into the same tube then transferred together by pippeting onto a new glass slide. These were gently covered with a cover slide.

When assaying for ascospores, rosettes were visualized using a Ziess microscope. Pictures were taken and processed using a Kodak DC290 camera and the Kodak Documentation System (MDS290). Per cross, two pictures (one at 160X + 50mm zoom and one at 400X + 50mm zoom) were taken of at least three representative rosettes. When assaying for *gfp*, rosettes were visualized at 400X using an Olympus BX51 and Olympus BH2 microscopes.

Quantification

For quantification of pigmented/unpigmented ascospores and ascospore shape, images were taken of rosettes at 63X magnification and all or a portion of the rosette was quantified. Numbers represent an average of at least 3 different rosettes with an average of >900 (± 200 standard deviation) ascospores counted per cross. For quantification of GFP in crosses, a line was drawn through clearly discernable asci on the bright-field image and the total number of asci quantified. On the GFP image, a line was drawn through any bright paired GFP spots originating from ascospores (post-meiotic signal after meiotic silencing resets). Marked bright-field and GFP images were merged. Asci marked in both pictures were subtracted from the total. Asci marked in the bright-field yet lacking GFP signal were counted as “GFP-off”, and those intersecting a GFP signal were counted as “GFP-on”. This was surely an overrepresentation of “GFP-off” since abortive meiosis and asci with GFP in a different focal plane than the ascus will be scored as GFP-off. Images from *spo11* and *ski8* crosses were taken at a

magnification, as described for *Rsp,* less than *mei-3*, as described for *gfp.* For *spo11* and *ski8* crosses, at least 3 rosettes were analyzed and an average of >170 asci (± 50 standard deviation) were counted per cross. For *mei-3* crosses, at least 8 rosettes were analyzed and an average of >110 asci (± 4 standard deviation) were counted per cross.

Contributions

Dong Whan Lee contributed several unpublished strains used in this work (see Table A1). Robert J. Pratt designed and performed all experiments and analyzed and interpreted the data. Rodolfo Aramayo advised on various aspects of experimentation. R.J.P. and R.A. wrote the manuscript.

CHAPTER IV

SMS-4, THE NEUROSPORA ORTHOLOG OF MAMMALIAN ELG PROTEIN, IS A NUCLEAR PROTEIN REQUIRED FOR MEIOTIC SILENCING BY UNPAIRED DNA BUT NOT FOR MEIOSIS

INTRODUCTION

Meiotic silencing is most likely an RNAi mechanism given that many of the genes required for the process are homologous to conserved RNAi machinery in Neurospora and other organisms. Specifically, there are an RdRP, *Sad-1*, an Argonaute, *Sms-2,* and a Dicer, *Sms-3*. All of these components localize outside of the nucleus along the nuclear periphery during meiotic prophase I. Another component, *Sad-2*, encodes a perinuclear protein required for localization of *Sad-1* to the nuclear periphery. The role of *Sad-2* in RNA silencing in other organisms is unknown. Mutations in any of the corresponding genes prevent formation of a significant number of viable progeny in homozygous mutant crosses. This observation suggests that meiotic silencing might be required for meiosis or ascus development (Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu and Metzenberg 2002; Shiu *et al.* 2006; Shiu *et al.* 2001).

Here we describe a new gene, *Sms-4*, required for meiotic silencing that localizes to the nucleus, the presumed site of meiotic *trans*-sensing and unpaired DNA. While required for meiotic silencing by unpaired DNA, *Sms-4*, is not required for meiosis or ascus development suggesting that the RNAi related components required for meiotic silencing likely have additional, independent roles in the meiosis or ascus development.

RESULTS

Mutagenesis strategy for identifying genes required for meiotic silencing

To elucidate the mechanism of meiotic *trans*-sensing and meiotic silencing, the following mutagenesis strategy was used to identify genes required for these

pathways. A male containing a deletion of the gene *Asm-1* and an ectopic, non-functional insertion of *Asm-1* was exposed to UV radiation and then used to fertilize a wild-type female. The resulting diploid nucleus of this mating contained two unpaired copies of *Asm-1*, which resulted in meiotic silencing of the only functional version. When *Asm-1* is silenced, a crop of white, inviable ascospores is produced (Aramayo and Metzenberg 1996). Dominant mutations that block meiotic silencing would prevent silencing of *Asm-1* and would therefore produce viable progeny. Viable progeny were selected for then screened in crosses containing an unpaired copy of the reporter gene *Round spore* (*Rsp*) to screen against, suppressors of *Asm-1*, "escapees" that result from meiotic silencing that is not 100% efficient, and wild-type siblings sheltered by dominant mutants defective in meiotic silencing. Strains that failed to silence unpaired *Rsp* were then tested for their ability to complement the sexual defects caused by the inactivation of known components of meiotic silencing. Mutant loci fitting these criteria were named *Suppressor of Meiotic Silencing* (*Sims*). Similarly behaving loci are named *Suppressor of Ascus Dominance* (*Sad*) by another group (Shiu *et al.* 2006; Shiu *et al.* 2001). This work focuses on the characterization of a mutation that defines a new complementation group we call *Suppressor of meiotic silencing-4* (*Sms-4*).

Meiotic silencing by unpaired DNA is not required for meiosis

It became immediately apparent when we began working with the *Sms-4*^{UV} allele that, unlike all previously identified mutations that eliminate meiotic silencing, it did not block meiosis. To characterize the development in the *Sms-4* mutant in more detail, we dissected fruiting bodies (perithecia) of Neurospora and observed the developing tissues for several days following fertilization. To monitor meiotic silencing in these crosses, we analyzed the silencing of an unpaired *hH1*$^{+}$::*gfp*$^{+}$ fusion gene relative to a paired fusion gene (Figure 16).

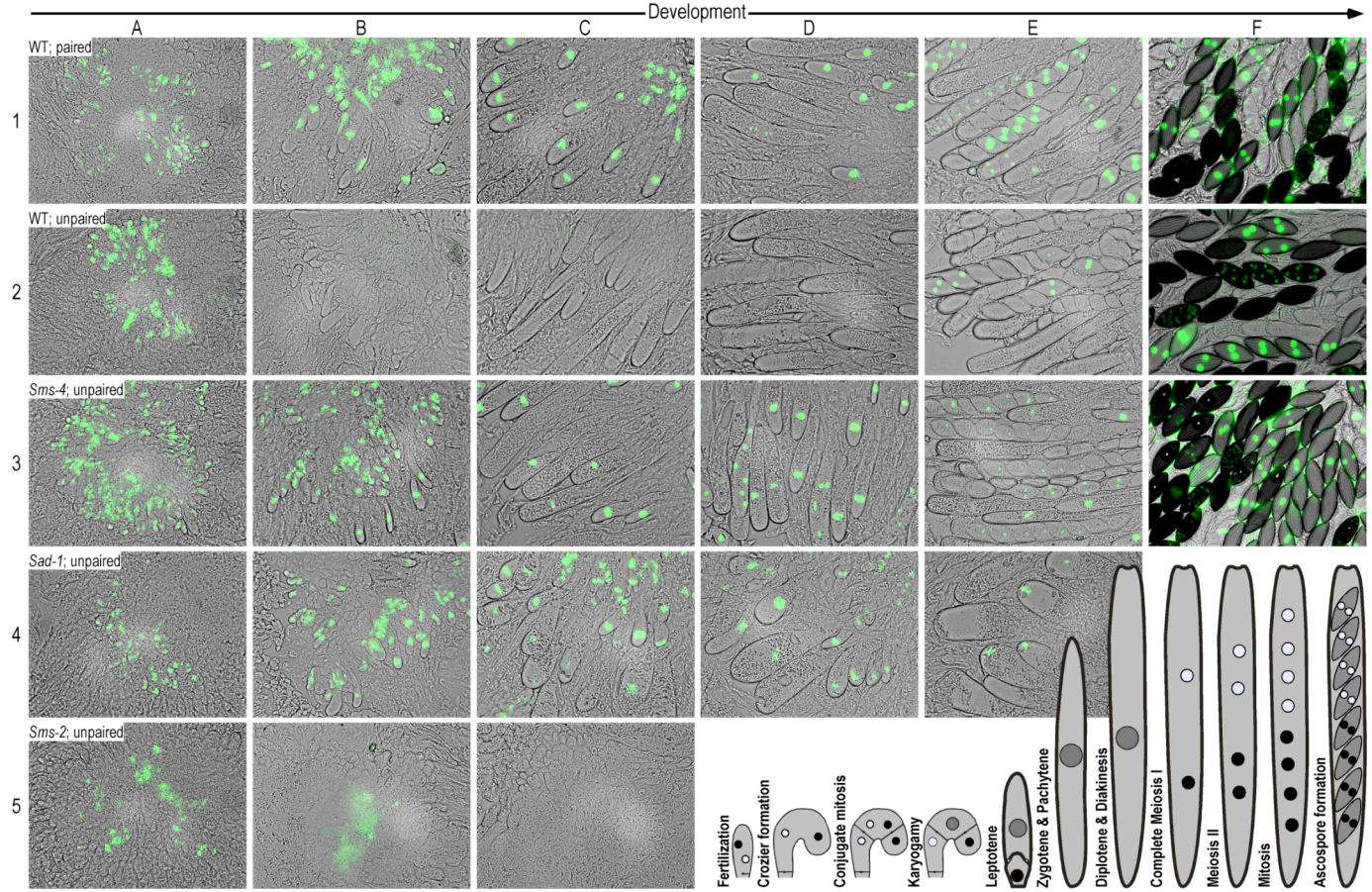

Figure 16. *Sms-4* is required for meiotic silencing by unpaired DNA but is not required for meiosis. Sexual tissues dissected from fruiting bodies (perithecia) were photographed at 400X magnification at different times after fertilization. Each row represents a different cross (Crosses 1 to 5, Table 4). Each column represents the same time following fertilization for all of the crosses. The pictures merge fluorescent (green) and bright-field (gray) images, where green represents the signal from a HH1::GFP fusion. The relevant genotypes and the pairing status of $hH1^{+}$::gfp^{+} in each cross is indicated in the first picture of each row. A cartoon diagram of ascus development and meiosis in *Neurospora crassa* based on Raju (1980) is shown at the lower right (not drawn to scale).

Table 4. Crosses performed during the characterization of *Sms-4*

Cross Number	Figure	Strains[a] Female	Male	Relevant Diploid Genotype[b]	Brief Description
01	16	RPNCR477A + H	RPNCR472A	*hH1*$^{+}$::*gfp*^{fs}/*hH1*$^{+}$::*gfp*$^{+}$	*hH1*$^{+}$::*gfp*$^{+}$ paired; wild-type
02	16	DLNCR246A + H	RPNCR472A	+/*hH1*$^{+}$::*gfp*$^{+}$	*hH1*$^{+}$::*gfp*$^{+}$ unpaired; wild-type
03	16	RPNCR232A + H	RPNCR416A	+/*hH1*$^{+}$::*gfp*$^{+}$; *Sms-4*^{UV}/*Sms-4*^{UV}	*hH1*$^{+}$::*gfp*$^{+}$ unpaired; *Sms-4* mutant
04	16	RPNCR237A + H	RPNCR487A	+/*hH1*$^{+}$::*gfp*$^{+}$; *Sad-1*$^{\Delta}$/*Sad-1*$^{\Delta}$	*hH1*$^{+}$::*gfp*$^{+}$ unpaired; *Sad-1* mutant
05	16	RPNCR238A + H	RPNCR475A	+/*hH1*$^{+}$::*gfp*$^{+}$; *Sms-2*^{RIP88}/*Sms-2*^{RIP88}	*hH1*$^{+}$::*gfp*$^{+}$ unpaired; *Sms-2* mutant
06	18A	DLNCR93A X RANCR06A		+/*Rsp*^{RIP93}	*Rsp* silencing; wild-type
07	18A	RPNCR250A X RPNCR142A		+/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; *Sms-4*^{UV}
08	18A	RPNCR200A X RPNCR201A		+/*Rsp*^{RIP93}; NCU01309$^{\Delta}$/ NCU01309$^{\Delta}$	*Rsp* silencing; NCU01309$^{\Delta}$
09	18A	RPNCR203A X RPNCR204A		+/*Rsp*^{RIP93}; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	*Rsp* silencing; Sms-4$^{\Delta}$ (NCU01310$^{\Delta}$)
10	18A	RPNCR206A X RPNCR207A		+/*Rsp*^{RIP93}; NCU01310$^{\Delta}$/ NCU01310$^{\Delta}$	*Rsp* silencing; NCU01311$^{\Delta}$
11	18A	RPNCR223A X RPNCR224A		+/*Rsp*^{RIP93}; *Intergenic region-1*^{RIP}/*Intergenic region-1*^{RIP}	*Rsp* silencing; *Intergenic region-1*^{RIP}
12	18A	RPNCR225A X RPNCR226A		+/*Rsp*^{RIP93}; *Intergenic region-2*^{RIP}/*Intergenic region-2*^{RIP}	*Rsp* silencing; *Intergenic region-2*^{RIP}
13	18A	RPNCR178A X RPNCR208A		+/*Rsp*^{RIP93}; *rca-1*$^{\Delta}$/*rca-1*$^{\Delta}$	*Rsp* silencing; *rca-1*$^{\Delta}$
14	18C	RPNCR232A X RPNCR283A		*his-3*::*hph*$^{+}$::*tk*$^{+}$/*his-3*::*hph*$^{+}$::*tk*$^{+}$; +/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; no insert to complement *Sms-4*^{UV}
15	18C	DLNCR246A X RPNCR248A		*his-3*::*hph*$^{+}$::*tk*$^{+}$/*his-3*::*hph*$^{+}$::*tk*$^{+}$; +/*Rsp*^{RIP93}	*Rsp* silencing; no insert to complement but *sms-4*$^{+}$
16	18C	RPNCR251A X RPNCR267A		*his-3*$^{+}$::*sms-4*$^{+}$/*his-3*$^{+}$::*sms-4*$^{+}$; +/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; wild-type insert to complement *Sms-4*^{UV}
17	18C	RPNCR280A X RPNCR267A		*his-3*$^{+}$::*sms-4*$^{+}$/*his-3*$^{+}$::*sms-4*$^{+}$; +/*Rsp*^{RIP93}; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	*Rsp* silencing; wild-type insert to complement *Sms-4*$^{\Delta}$

Table 4. Continued

Cross Number	Figure	Strains[a] Female	Male	Relevant Diploid Genotype[b]	Brief Description
18	18C	RPNCR286A X	RPNCR287A	*his-3*$^{+}$::*sms-4*^{W97A}/*his-3*$^{+}$::*sms-4*^{W97A}; +/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; W97A mutant insert to complement *Sms-4*^{UV}
19	18C	RPNCR336A X	RPNCR338A	*his-3*$^{+}$::*sms-4*^{W97*}/*his-3*$^{+}$::*sms-4*^{W97*}; +/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; W97* mutant insert to complement *Sms-4*^{UV}
20	18C	RPNCR336A X	RPNCR287A	*his-3*$^{+}$::*sms-4*^{W97A}/*his-3*$^{+}$::*sms-4*^{W97*}; +/*Rsp*^{RIP93}; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; W97A and W97* mutant inserts to complement *Sms-4*^{UV}
21	19A	RANCR49A	RANCR06A	+/+	For Northern, *sms-4*$^{+}$
22	19A	RPNCR321A	RPNCR203A	*Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	For Northern, *Sms-4*$^{\Delta}$
23	19A	RANCR49A	RPNCR73A	+/*Rsp*^{RIP93}	For Northern, *sms-4*$^{+}$ with *Rsp* unpaired
24	19A	RPNCR321A	RPNCR244A	+/*Rsp*^{RIP93}; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	For Northern, *Sms-4*$^{\Delta}$ with *Rsp* unpaired
25	19G	RPNCR506A	FGSC 2490	*sms-4*$^{+}$::*gfp*$^{+}$/+	To analyze sms-4::gfp expression in paraphyses
26		RPNCR500A	FGSC 2490	*sms-4*$^{+}$::*gfp**/+	*sms-4*$^{+}$::*gfp** control for cross 25
27	19H-L	RPNCR531A	RPNCR505A	*Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	sms-4::gfp expression during meiosis
28	19H-L	RPNCR531A	RPNCR508A	*rid-1*^{RIP}/*rid-1*^{RIP}; *Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	sms-4::gfp expression during meiosis
29		RPNCR531A	RPNCR509A	*Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp**	*sms-4*$^{+}$::*gfp** control for crosses 27 and 28
30	21A	RPNCR202A X	RPNCR203A	*Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	Control for spore morphology in *Sms-4* mutant
31	21A	RPNCR204A X	RPNCR242A	*Rsp*^{RIP93}/*Rsp*^{RIP103}; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	Control for spore morphology in *Sms-4* and *Rsp* mutants
32	21A	RANCR05A X	RPNCR173A	+/*Rsp*^{RIP103}	Methylated *Rsp*^{RIP103} – Homeologous inducer, *cis*-silencing, spore phenotype

Table 4. Continued

Cross Number	Figure	Strains[a] Female	Male	Relevant Diploid Genotype[b]	Brief Description
33	21A	RANCR05A X	RPNCR242A	+/*Rsp*^{RIP103}; +/*Sms-4*$^{\Delta}$	Methylated *Rsp*^{RIP103}; *Sms-4*$^{\Delta}$ heterozygous
34	21A	RPNCR78A X	RPNCR103A	+/*Rsp*^{RIP103}; *dim-2*/*dim-2*	Demethylated *Rsp*^{RIP103}
35	21A	RPNCR78A X	RPNCR243A	+/*Rsp*^{RIP103}; +/*Sms-4*$^{\Delta}$; *dim-2*/*dim-2*	Demethylated *Rsp*^{RIP103}; *Sms-4*$^{\Delta}$ heterozygous
36	21A	RPNCR202A X	RPNCR243A	+/*Rsp*^{RIP103}; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	Methylated *Rsp*^{RIP103}; *Sms-4*$^{\Delta}$ homozygous
37	21A	RANCR06A X	RPNCR212A	+/*Rsp*$^{\Delta}$	*Rsp*$^{\Delta}$ – indel inducer, *cis*-silencing, spore phenotype
38	21A	RPNCR203A X	RPNCR240A	+/*Rsp*$^{\Delta}$; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	*Rsp*$^{\Delta}$; *Sms-4*$^{\Delta}$ homozygous
39	21A	S1 a X	RPNCR241A	+/*Rsp*$^{\Delta}$; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp*$^{\Delta}$; *Sms-4*^{UV} homozygous
40	21B	FGSC 2490	FGSC 8329	+/*mat-A*(IL->VR)	*mat-A*(IL->VR) – indel inducer, *cis*-silencing, ascus phenotype
41	21B	FGSC 8741	FGSC 8329	*Sad-1*$^{\Delta}$/+; +/*mat-A*(IL->VR)	*mat-A*(IL->VR); *Sad-1*$^{\Delta}$ heterozygous
42	21B	RPNCR158A	FGSC 8329	+/*mat-A*(IL->VR); *Sms-4*^{UV}/+	*mat-A*(IL->VR); *Sms-4*^{UV} heterozygous
43	21A	DLNCT291A	DLNCR245A	*his-3*$^{+}$::*asm-1*$^{+}$/*his-3*::*hph*$^{+}$::*tk*$^{+}$/	*Asm-1*^{ect} – indel inducer of *trans*-silencing, spore phenotype
44	21A	RPNCR253B	RPNCR220A	*his-3*$^{+}$::*asm-1*$^{+}$/*his-3*::*hph*$^{+}$::*tk*$^{+}$/; *Sms-4*$^{\Delta}$/*Sms-4*$^{\Delta}$	*Asm-1*^{ect}; *Sms-4*$^{\Delta}$ homozygous
45	21A	RPNCR253B	RPNCR279A	*his-3*$^{+}$::*asm-1*$^{+}$/*his-3*$^{+}$::*asm-1*$^{+}$; *Sms-4*$^{\Delta}$/*Sms-4*^{UV}	Control for spore color in *Sms-4* mutant
46	21B	DLNCR246A	FGSC 8753	*his-3*::*hph*$^{+}$::*tk*$^{+}$/*his-3*$^{+}$::*hH3*::*hH4-1*	*hH3hH4-1*^{ect} – indel inducer of *trans*-silencing, ascus phenotype
47	21B	RPNCR237A	FGSC 8753	*Sad-1*$^{\Delta}$/+; *his-3*::*hph*$^{+}$::*tk*$^{+}$/*his-3*$^{+}$::*hH3*::*hH4-1*	*hH3hH4-1*^{ect}; *Sad-1*$^{\Delta}$ heterozygous
48	21B	RPNCR232A	FGSC 8753	*his-3*::*hph*$^{+}$::*tk*$^{+}$/*his-3*$^{+}$::*hH3*::*hH4-1*; *Sms-4*^{UV}/+	*hH3hH4-1*^{ect}; *Sms-4*^{UV} heterozygous

Table 4. Continued

Cross Number	Figure	Strains[a] Female	Male	Relevant Diploid Genotype[b]	Brief Description
49[c]	23A	RPNCR555A	RPNCR558A	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	Localization of sms-4::gfp & RFP::SAD-2
50	23B	RPNCR531A	RPNCR505A	*Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	Localization of sms-4::gfp; wild-type
51	23B	RPNCR586A	RPNCR521A	*Sad-1*$^{\Delta}$/*Sad-1*$^{\Delta}$; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	Localization of sms-4::gfp; *Sad-1* mutant
52	23B	RPNCR533A	RPNCR522A	*sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$; *Sad-2*^{RIP}/*Sad-2*^{RIP}	Localization of sms-4::gfp; *Sad-2* mutant
53	23B	RPNCR531A	RPNCR523A	+/*his-3*$^{+}$::*rfp*::*sms-2; Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$; +/*Sms-2*^{RIP}	Localization of sms-4::gfp; *Sms-2* mutant
54	23B	RPNCR531A	RPNCR560A	+/*his-3*$^{+}$::*rfp*::*sms-3; Rsp*^{RIP93}/+; +/*Sms-3*^{RIP}*; sms-4*$^{+}$::*gfp**/*sms-4*$^{+}$::*gfp*$^{+}$	Localization of sms-4::gfp; *Sms-3* mutant
55	24A	RPNCR548A	RPNCR549A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$	Localization of sad-1::gfp; wild-type
56	24B	RPNCR573A	RPNCR574A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$; *Sad-2*^{RIP}/*Sad-2*^{RIP}	Localization of sad-1::gfp; *Sad-2* mutant
57	24C	RPNCR575A	RPNCR576A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$; *mei-3*/*mei-3*	Localization of sad-1::gfp; *mei-3* mutant
58	24D	RPNCR552A	RPNCR553A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$; *Sms-4*^{UV}/*Sms-4*^{UV}	Localization of sad-1::gfp; *Sms-4* mutant
59[c]	24E	RPNCR552A	RPNCR557A	*sad-1*$^{+}$::*gfp*$^{+}$/+; +/*his-3*$^{+}$::*rfp*$^{+}$::*sad-2; Sms-4*^{UV}/*Sms-4*^{UV}	Localization of sad-1::gfp & RFP::SAD-2*; Sms-4* mutant
60	17	RPNCR587A	RPNCR487A	*Sad-1*$^{\Delta}$/*Sad-1*$^{\Delta}$; *hH1*$^{+}$::*gfp*^{fs}/*hH1*$^{+}$::*gfp*$^{+}$	*hH1*$^{+}$::*gfp*$^{+}$ paired; *Sad-1* mutant
61	17	RPNCR588A	RPNCR475A	*Sms-2*^{RIP}/*Sms-2*^{RIP}; *hH1*$^{+}$::*gfp*^{fs}/*hH1*$^{+}$::*gfp*$^{+}$	*hH1*$^{+}$::*gfp*$^{+}$ paired; *Sms-2* mutant
62	20A	RPNCR570A	RPNCR508A	*Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp*$^{+}$/*sms-4*$^{+}$::*gfp*$^{+}$	*Rsp* silencing; *sms-4*$^{+}$::*gfp*$^{+}$
63	20B	RPNCR570A	RPNCR370A	*Rsp*^{RIP93}/+; *sms-4*$^{+}$::*gfp*$^{+}$/*Sms-4*^{UV}	*Rsp* silencing; *sms-4*$^{+}$::*gfp*$^{+}$; *Sms-4*^{UV}
64	20C	RPNCR572A	RPNCR549A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$; *Rsp*^{RIP93}/+	*Rsp* silencing; *sad-1*$^{+}$::*gfp*$^{+}$

Table 4. Continued

Cross Number	Figure	Strains[a] Female	Male	Relevant Diploid Genotype[b]	Brief Description
65	20D	RPNCR572A	RPNCR553A	*sad-1*$^{+}$::*gfp*$^{+}$/*sad-1*$^{+}$::*gfp*$^{+}$; *Rsp*^{RIP93}/+; *Sms-4*^{UV}/*Sms-4*^{UV}	*Rsp* silencing; *sad-1*$^{+}$::*gfp*$^{+}$; *Sms-4*^{UV}
66	20E	RLM 30-12	RPNCR410A	+/*his-3*::*hph*$^{+}$::tk^{+}; *Sad-2*^{RIP}/*Sad-2*^{UV}	*Sad-2* mutant phenotype
67	20F	RLM 24-25	RPNCR410A	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*::*hph*$^{+}$::tk^{+}; *Sad-2*^{RIP}/*Sad-2*^{UV}	A single copy *rfp*$^{+}$::*sad-2* to complement *Sad-2* arrest
68	20G	RLM 24-25	RLM 25-25	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*; *Sad-2*^{RIP}/*Sad-2*^{RIP}	Two copies *rfp*$^{+}$::*sad-2* to complement *Sad-2* arrest
69	20H	RPNCR571A	RPNCR410A	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*::*hph*$^{+}$::tk^{+}; *Rsp*^{RIP93}/+; *Sad-2*^{RIP}/*Sad-2*^{UV}	A single copy *rfp*$^{+}$::*sad-2* to silence *Rsp*
70	20I	RPNCR571A	RLM 25-25	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*; *Rsp*^{RIP93}/+; *Sad-2*^{RIP}/*Sad-2*^{RIP}	Two copies *rfp*$^{+}$::*sad-2* to silence *Rsp*
71	20J	RPNCR571A	RPNCR269A	*his-3*$^{+}$::*rfp*$^{+}$::*sad-2*/*his-3*::*hph*$^{+}$::tk^{+}; *Rsp*^{RIP93}/rsp^{RIP94}; *Sad-2*^{RIP}/+	A single copy *rfp*$^{+}$::*sad-2;* no functional *Rsp*

[a]Crosses were non-directional, unless otherwise indicated. "+ H" indicates that the strain was a heterokaryon with helper-strain FGSC 4564. Heterokaryons were used to normalize female-fertility among mutant strains. Although FGSC 4564 can readily complement any pre-dikaryotic defects, the mating type a^{m1} mutant allele present in this nucleus prevents it from entering ascogenous tissue, thus blocking its participation in meiosis (Perkins 1984)

[b]Complete genotypes are described in Table A1.

[c]In these crosses, tissues were analyzed with and without fixation by formaldehyde cross-linking. Regardless of fixation, the sub-cellular localization of the fusion proteins studied did not change in these tissues. Localization of all other fusion proteins was analyzed in unfixed cells.

For comparison, wild-type, *Sad-1* mutant and *Sms-2* mutant crosses were also analyzed. All crosses performed in this study are listed in Table 4.

hH1^{+}::*gfp*$^{+}$ is a good reporter for meiotic silencing for a few reasons. First, *hH1* is dispensable for meiosis (Folco *et al.* 2003). Also, the fusion protein is readily detectable by fluorescent microscopy in most cell types, and the protein conveniently localizes to the nuclei, allowing the monitoring of nuclear division. However, these same attributes cause problems when the female contains a *hH1*$^{+}$::*gfp*$^{+}$ fusion, since the intense signal from the maternal tissues creates a high background. Therefore, we engineered an allele of the fusion gene that contains a frameshift in the linker region between the *hH1*$^{+}$ and *gfp*$^{+}$ genes (*hH1*$^{+}$::*gfp*^{fs}). When present in the female, this construct can pair with the *hH1*$^{+}$::*gfp*$^{+}$ allele contributed by the male, allowing the monitoring of HH1::GFP expression during development. When either paired or unpaired, since only the male contributes a functional *hH1*$^{+}$::*gfp*$^{+}$, all fluorescent signal must derive from nuclei participating in sex.

The pattern of sexual development in Neurospora is well documented (Raju 1980) and diagrammed in Figure 16. The developmental stages can be followed by observing ascus morphology and the number of nuclei per ascus. The sexual development and *hH1*$^{+}$::*gfp*$^{+}$ expression of wild-type crosses carrying paired *gfp*$^{+}$ DNA served as a control (Figure 16, 1A-1F). Following fertilization, male and female nuclei propagate in a heterokaryotic tissue (Figure 16, 1A). Cells carrying nuclei of both mating types then form dikaryotic, or ascogenous, tissue. The primordial specialized crozier cells form from this tissue. Each crozier initially carries only one nucleus of each mating type. Following a coordinated mitosis and the formation of two septa, croziers develop into a three-celled structure containing a uninucleate basal cell, a dikaryotic middle cell (i.e., ascus mother cell) and a uninucleate tip cell.

Following karyogamy, the ascus mother cell forms the only known diploid cell in Neurospora. The diploid nucleus undergoes meiosis. As the meiotic asci mature, they become readily visible and distinguishable from the maternal paraphysal hyphae (Figure 16, 1B). The subsequent elongation of the ascus to its maximal length marks the meiotic stages (leptotene to pachytene) at which chromosomes align, recombine and synapse (Figure 16, 1B to 1C). As the tip of the ascus becomes flattened, the formation of a pore marks the developmental stage (diplotene and diakinesis) in which the synaptonemal complex dissolves and homologs prepare for segregation (Figure 16, 1C and 1D). Successive stages are defined by the number of nuclei present in each ascus (i.e., two following Meiosis I, four following Meiosis II, and eight following the post meiotic mitosis (Figure 16, 1D and 1E). Finally, the deposition of membrane components around the nuclei initiates the process of cellularization and ascospore maturation. Inside each ascospore, nuclei undergo yet another division prior to melanization then GFP signal is either diminished or lost (Figure 16, 1E and 1F).

HH1^{+}::*gfp*$^{+}$ expression of wild-type crosses carrying unpaired *gfp*$^{+}$ DNA served as a control for normal silencing of this reporter gene (Figure 16, 2A to 2F). As expected, ascus development was normal in crosses involving unpaired *gfp*$^{+}$ DNA (Figure 16, 2A to 2F). However, although the GFP signal was readily visible in early heterokaryotic tissues (Figure 16, 2A), it disappeared from all ascogenous tissues following the appearance of the first meiotic asci (Figure 16, 2B to 2E). This loss of GFP signal was interpreted as a combined consequence of meiotic silencing preventing the production of new HH1::GFP protein and the normal turn-over of pre-meiotic HH1::GFP protein. Meiotic silencing persisted until after ascospores formed (Figure 16, 2E and 2F), as

previously described for the *hH1*$^{+}$::*gfp*$^{+}$ expressed under a heterologous promoter (Freitag *et al.* 2004a).

Developmental defects were obvious in crosses homozygous for mutant alleles of *Sad-1* and *Sms-2* (Figure 16, Crosses 4 and 5). *Sad-1* mutants arrested in Meiosis I, as evidenced by the single GFP dot observed in all asci (Figure 16, 4B to 4E). As expected, GFP signal was still detected in most of the asci, demonstrating the lack of meiotic silencing of the unpaired reporter DNA in this mutant. Although the presence of GFP signal in the *Sad-1* mutant could, in principle, be the result of meiotic arrest rather than loss of meiotic silencing, this alternative is unlikely given that early pre-arrested asci produced signal (Figure 16, compare 2B with 4B). Consistent with this interpretation, Neurospora *meiosis-3* (*RecA*) mutants arrest at a similar meiotic stage, yet still silence the same reporter gene efficiently (Chapter III). As the *Sad-1* asci aged, some of them lost GFP signal (Figure 16, 4D and 4E), perhaps due to cell death.

With *Sms-2*, we observed an earlier developmental defect (Figure 16, 5A to 5C). Although GFP signal was initially present in heterokaryotic tissues (Figure 16, 5A), at later time points only a diffuse signal could be seen (Figure 16, 5B) and eventually all GFP signal was lost (Figure 16, 5C). No meiotic asci were observed in these crosses, suggesting a developmental arrest prior to or immediately following, karyogamy. Why GFP signal was eventually lost in *Sad-1* and *Sms-2* mutants remains unclear but could be due to down-regulation of the *hH1*$^{+}$ gene or to cell death. Based on control crosses (Figure 17, Crosses 60 and 61), the loss of signal was not due to establishment of *Sad-1*- or *Sms-2*-independent silencing by unpaired DNA, since the signal was also lost in *Sad-1* and *Sms-2* crosses where *hH1*$^{+}$::*gfp*$^{+}$ was paired.

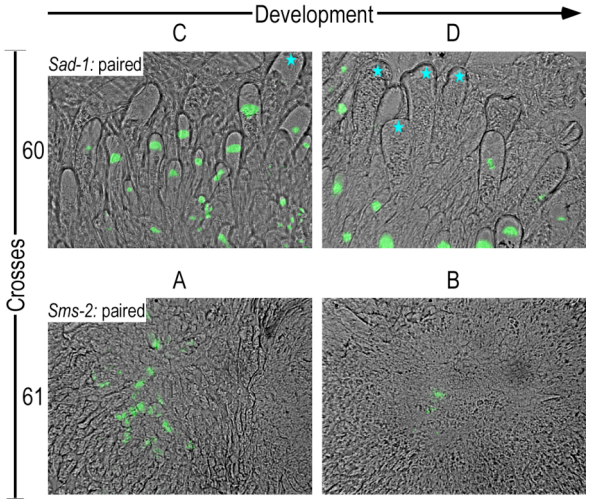

Figure 17. GFP signal was lost in tissues from *Sad-1* and *Sms-2* crosses even when *hH1*$^{+}$::*gfp*$^{+}$ was paired. Organization is the same as Figure 16. Blue stars designate the tips of meiotic asci that have lack GFP signal.

In contrast to these mutants in meiotic silencing, crosses between *Sms-4* mutants were developmentally indistinguishable from wild-type crosses (Figure 16, 3A to 3F). Furthermore, despite *h H1*$^{+}$::*gfp*$^{+}$ being unpaired, GFP signal was expressed as in the wild-type crosses in which *hH1*$^{+}$::*gfp*$^{+}$ was paired. This suggests that *Sms-4* is required for meiotic silencing to occur through the full course of meiosis. In this experiment, surprisingly we observed loss of signal in pre-karyogamic tissues of wild-type crosses but never in pre-karyogamic tissues of *Sad-1* or *Sms-4* crosses (Figure 16, compare 2B with 3B and 4B). This was unexpected, given the ascus-autonomous nature of meiotic silencing (Aramayo and Metzenberg 1996; Shiu and Metzenberg 2002). These results suggest that the spatial and temporal regulation of meiotic silencing should be reinvestigated. In summary, *Sms-4* is not required for meiosis, representing a new class of genes required for meiotic silencing.

SMS-4 is the ortholog of the mammalian mRNP component ELG

The original *Sms-4* mutation (*Sms-4*^{UV}) was mapped in two steps. First, it was genetically mapped to an ~500-kbp region located ~17 cM from *cycloheximide-2* (*cyh-2*) and ~5 cM from *inositol* (*inl*). Second, a strain containing the original *Sms-4*^{UV} mutation, flanked by the *cyh-2* and *inl* markers, was crossed to a polymorphic *Neurospora crassa* isolate (Mauriceville). Restriction fragment length polymorphisms (RFLPs) were used to map *Sms-4* in progeny that had a crossover between *cyh-2* and *inl*. In this way cosmid G23:G9 which spanned an apparent deletion of ~25-kbp present only in the progeny showing suppression, was identified. Genome annotation (Galagan *et al.* 2003) predicted the presence of four genes in the region (Figure 18).

To determine which genes, if any, were responsible for the suppressor phenotype, we performed a directed scanning mutagenesis of the region (Figure 18A). Three of the predicted genes were individually targeted by gene replacement. A deletion of the fourth gene, *rca-1,* was obtained from Dan Ebbole (Shen *et al.* 1998). RIP mutagenesis was used to inactivate two additional regions in the large intergenic region located between NCU01311 and *rca-1* incase the gene prediction algorithm missed any genes. Meiotic silencing was then tested in crosses homozygous for a given mutation in the region and containing unpaired *Rsp* DNA (i.e., *rsp*$^{+}$/*Rsp*^{RIP93})(Table 4, Crosses 6 to 13). Only the second predicted gene, NCU01310, showed suppression of meiotic silencing and was named *Sms-4*.

The gene structure of *Sms-4* was determined by mapping the 5'-end, 3'-end, and introns of the transcripts (Materials and Methods, Figure 18A). By BLAST analysis, the predicted amino acid sequence revealed two regions of sequence similarity to other proteins. These were termed Region A and Region B (Figure 18A). Region A is ~150 aa long (plus or minus a variable indel in the region)

Figure 18. SMS-4 is a possible RNA binding protein orthologous to mammalian ELG. A) Diagram of the *Sms-4* locus. The top grey box represents the cosmid G23:G9 that spans the *Sms-4*UV allele. Arrows represent the predicted genes in the region. Below, the relative position of the *Sms-4*UV allele and that of the different alleles constructed to test meiotic silencing are represented by solid or dashed bars corresponding to the regions deleted or mutated by RIP, respectively. Here, the allele names and the percentages of spindle-shaped ascospores produced by these mutants in homozygous crosses are indicated above and below the lines, respectively. Higher percentages indicate lower meiotic silencing. Enlarged below, the *Hin*dIII fragment used to complement the *Sms-4*UV allele is diagrammed (*Sms-4* locus). Boxes represent exons. The black and grey areas in exons correspond to Region A and Region B of the protein, respectively. The arrow and vertical bar indicate the predominant 5'-transcription start and 3'-polyadenylation sites, respectively. A diagram of the spliced *Sms-4* mRNA is presented below with a histogram produced by the alignment of the full-length SMS-4 orthologs from available ascomycetes. The height of the bars reflects the sequence conservation of the each region.
B) Sequence analysis of Region A. A multiple sequence alignment of Region A from diverse organisms is shown. The position of the first amino acid (left) and the name of the organism (right) are indicated. Above the alignment, a consensus histogram is shown. The asterisk signals the position of the conserved tryptophan 97 in SMS-4 that was mutated (Panel C). Below the alignment, the results from secondary structure predictions and similarity searches using the *S. pombe* ortholog are presented. Residues predicted to be in a beta sheets or alpha helices are colored in blue and red, respectively. Similarity scores between the structures (Jscores) are indicated at the left and the names of the different proteins tested are given at the right of the displayed sequences. The Jscore corresponds roughly to the number of C-alpha atoms of the model within 3.5 Å of the native structure (Ginalski *et al.* 2003). See Table 5 for details.
C) Complementation of *Sms-4* mutants. The chart shows the results of complementation experiments performed in the presence of unpaired *Rsp* DNA (*Rsp*RIP93*/rsp*+). The *Sms-4* alleles of the zygote at the *Sms-4* and ectopic *his-3* loci are indicated on the X-axis, corresponding to crosses 14 to 20. In the chart, NI indicates that there was no *Sms-4* insert at *his-3*. The resulting percentage of spindle-shaped ascospores quantified from each cross is given by the Y-axis. Crosses are listed in Table 4.

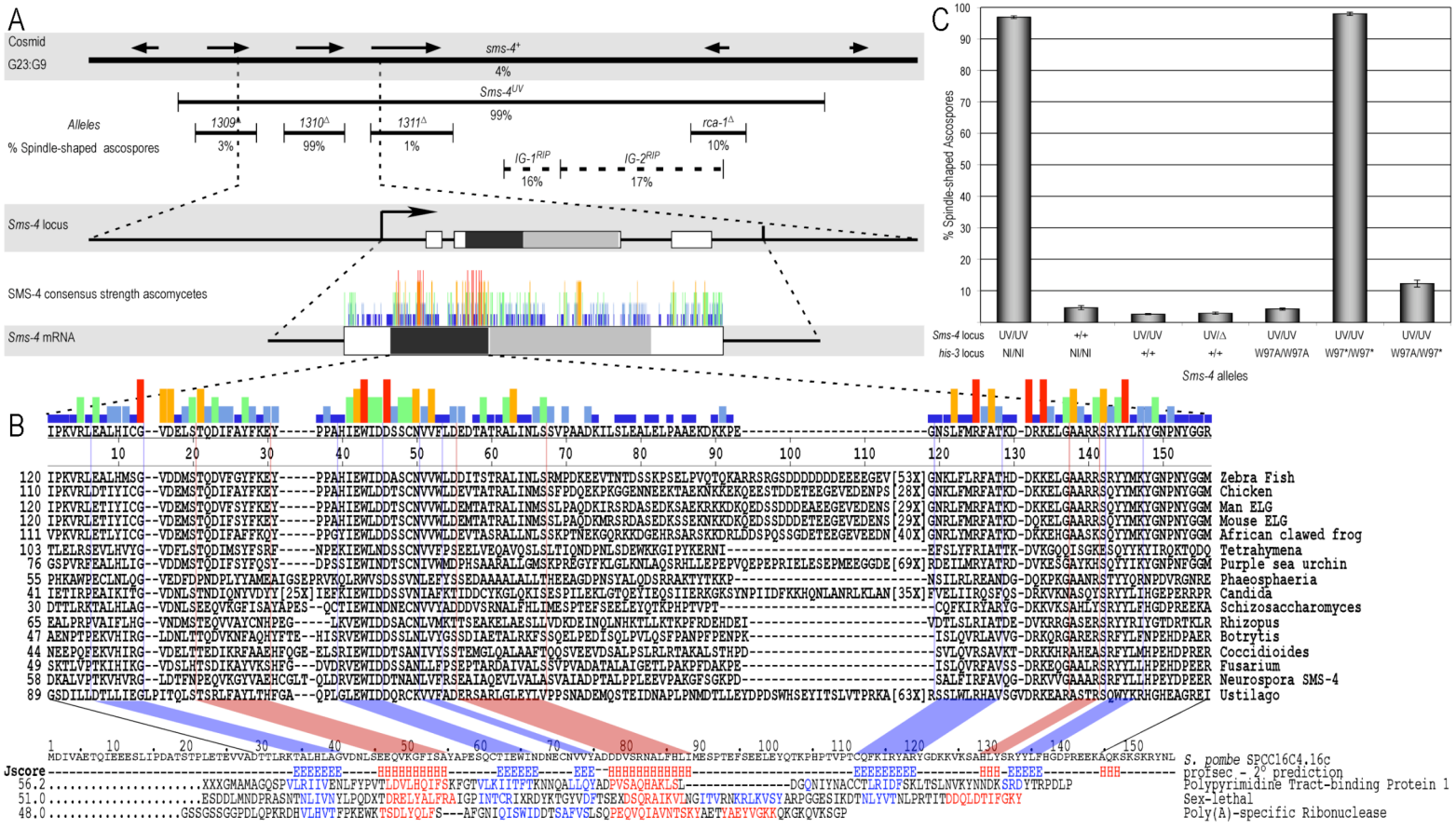
A
Cosmid
G23:G9
sms-4+
4%
Sms-4UV
99%
Alleles
% Spindle-shaped ascospores
1309Δ
3%
1310Δ
99%
1311Δ
1%
rca-1Δ
10%
IG-1RIP
16%
IG-2RIP
17%
Sms-4 locus
SMS-4 consensus strength ascomycetes
Sms-4 mRNA
B
C
% Spindle-shaped Ascospores
Sms-4 locus
his-3 locus
UV/UV
NI/NI
+/+
NI/NI
UV/UV
+/+
UV/Δ
+/+
UV/UV
W97A/W97A
UV/UV
W97*/W97*
UV/UV
W97A/W97*
Sms-4 alleles
Zebra Fish
Chicken
Man ELG
Mouse ELG
African clawed frog
Tetrahymena
Purple sea urchin
Phaeosphaeria
Candida
Schizosaccharomyces
Rhizopus
Botrytis
Coccidioides
Fusarium
Neurospora SMS-4
Ustilago
S. pombe SPCC16C4.16c
Jscore
profsec.- 2° prediction
Polypyrimidine Tract-binding Protein 1
Sex-lethal
Poly(A)-specific Ribonuclease

Table 5: SMS-4 and orthologs

Organism Scientific Name (Common Name)	Source [a]	Source ID number	% Identity Region A[b]
Aspergillus nidulans	Broad	AN0266.3	ND
Coccidioides immitis	Broad	CIMG_04801.2	66
Botrytis cinerea	Broad	BC1G_03701.1	66
Sclerotinia sclerotiorum	Broad	SS1G_12497.1	ND
Schizosaccharomyces pombe	NCBI	O74460	61
Chaetomium globosum	Broad	CHGG_02612.1	ND
Neurospora crassa	Broad	NCU01310.3	100
Magnaporthe grisea	Broad	MGG_06901.5	ND
Fusarium graminearum	Broad	FG01895.1	77
Phaeosphaeria nodorum	NCBI	EAT87599.1	61
Yarrowia lipolytica	NCBI	XP_500259.1	ND
Danio rerio (Zebra fish)	NCBI	NP_956437.1	29
Gallus gallus (Chicken)	NCBI	NP_001012823.1	40
Homo sapiens (Man)	NCBI	hmm51052	40
Mus musculus (Mouse)	NCBI	NP_080094.2	40
Xenopus laevis (African clawed frog)	NCBI	AAH77240.1	33
Tetrahymena thermophila	NCBI	XP_001028053.1	56
Strongylocentrotus purpuratus (Purple sea urchin)	NCBI	XP_001184676.1	19
Rhizopus oryzae	Broad	RO3G_17042.1	60
Ustilago maydis	Broad	UM03770.1	24
Candida albicans	NCBI	XP_720645.1	30

[a]NCBI = National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov/>, Broad = Broad Institute <http://www.broad.mit.edu/>

[b]% Identity in Region A based on alignment presented in Figure 18B. ND = not determined.

corresponding to PFAM-B 22455, a motif of unknown function. It has been identified in fungi, aveolata, green algae and metazoa, primarily in hypothetical proteins (Figure 18B, Table 5 and data not shown). In mammals, this region is only present in the ELG protein, which has no known function but was recently isolated as part of the mRNP, the complex of proteins that are associated with nascent mature mRNA (Merz *et al.* 2007). Notably, Region A is apparently absent from terrestrial plants and several model organisms, namely *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*.

The SMS-4 ortholog of *Schizosaccharomyces pombe*, SPCC16C4.16c, contains only Region A, and thus likely represents the minimal functional peptide. To gain further insight into the possible function of this region, the *S. pombe* ortholog

was analyzed using several programs that use predicted secondary structure to search for proteins of similar structure (see Materials and Methods) (Bujnicki *et al.* 2001; Ginalski *et al.* 2005). Interestingly, weakly significant hits were obtained to three different RNA-binding proteins: polypyrimidine tract-binding protein, sex-lethal and poly(A)-specific ribonuclease (Figure 18B, lower alignment), corresponding to the RNA recognition motif of the former two and to an uncharacterized region of the latter.

Region B is ~190 aa long, highly polar and arginine-rich, and shows weak similarity to a number of proteins in a BLAST search; the ones of predicted function are proposed to perform various functions in RNA metabolism. The amino acid sequence of the different proteins were unalignable, meaning that different residues were shared between the different BLAST hits of SMS-4. Apparently, therefore, the low complexity and arginine-richness of this region is the characteristic shared among the different proteins that were recognized. In support of this conjecture, the amino acid sequence of this region was even poorly conserved among fellow ascomycetes (Figure 18A, histogram).

To unequivocally determine the involvement of *Sms-4* in meiotic silencing and to identify the contributions to meiotic silencing of the other genes in the region, we performed a complementation analysis. The loss of meiotic silencing in the original *Sms-4*^{UV} mutant was fully complemented by inserting a copy of the *Sms-4* gene ectopically at the *his-3* locus of both parents (Figure 18C, Cross 16). Also, the ectopic copies complemented in a cross *trans*-heterozygous for *Sms-4* at the *Sms-4* locus; in which the entire region in the original *Sms-4*^{UV} allele was unpaired except for *Sms-4*, demonstrating that no other genes removed by the original deletion contribute significantly to the suppression phenotype (Figure 18C, Cross 17). The absolute conservation of the tryptophan 97 aa in Region A (W97) suggests that this residue plays a functional role.

However, an SMS-4 with an alanine substitution at this conserved tryptophan residue (W97A), still performed normal silencing as induced by *Rsp*RIP93 (Figure 18C, Cross 18). A mutant version of the protein containing both a stop mutation and a frameshift in the region (W97*) could not complement (Figure 18C, Cross 19) suggesting that the coding sequence of *Sms-4* was important for silencing. The W97A allele was still able to complement meiotic silencing, even when only one copy was present (Figure 18C, Cross 20).

In summary, the loss of meiotic silencing observed as a result of the Sms-4UV allele was likely due to the loss of SMS-4, a conserved protein orthologous to the mammalian ELG and is predicted to bind RNA.

SMS-4 is more or less ubiquitously expressed during the Neurospora life cycle

To gain insights into the role of *Sms-4* in the Neurospora life cycle, we performed a Northern analysis of Neurospora cultures grown under different conditions (Figure 19A). *Sms-4* transcript was detected under all standard culturing conditions tested, but it represented a higher portion of the total RNA during growth on solid media (Figure 19A, compare lanes 1 and 3 with 5 and 7), particularly under conditions conducive to female development (Figure 19A, compare lane 5 to 7 and 9). *Sms-4* transcript was also detected in fertilized cultures undergoing sex in the presence or absence of unpaired DNA (Figure 19A, lanes 11 and 13).

Fertilized cultures are a complex mixture of cell types: some cells differentiate because of fertilization while others remaining unfertilized. If a gene is expressed significantly higher following fertilization, e.g., *spo11*$^{+}$, the change in expression should be detectable under these culturing conditions (Figure 19A, middle panel). For genes expressed prior to fertilization, e.g., *Sms-4*, it is impossible to

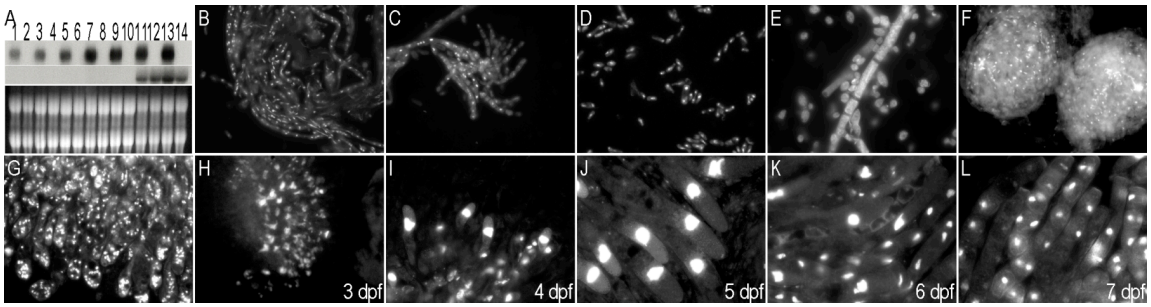

Figure 19. Expression of *sms-4*$^+$. A) Northern analysis of *sms-4*$^+$ under various growth conditions. RNA was extracted from strains grown as follows: liquid Vogel's medium (lanes 1 and 2), liquid Westergaard's medium (lanes 3 and 4), solid Vogel's medium (lanes 5 and 6), solid Westergaard's medium (lanes 7 and 8), solid Westergaard's medium then mock fertilized (lanes 9 and 10), and solid Westergaard's medium then fertilized to produce zygotes without (lanes 11 and 12) or with (lanes 13 and 14) unpaired *Rsp* DNA. Vogel's media are nitrogen-rich and promote asexual development. In contrast, Westergaard's media are nitrogen-poor and conducive to female sexual development, particularly when solid (see Materials and Methods for details). RNA samples from *sms-4*$^+$ wild type and from *Sms-4* mutant strains were loaded in odd and even number lanes, respectively. The *spo11* probe was included as a control for the relative level of meiotic RNA obtained in the different extractions (middle panel). The ethidium bromide stained gel served as a loading control (bottom panel). B to L) Expression of SMS-4::GFP during the Neurospora life cycle. Vegetative cell-types (B to E) and sexual tissues (F to L) were photographed at 600X magnification. Panels B to E correspond to vegetative haploid development: mycelia (Panel B), macroconidiophores (C), young blastoconidia (D), and older blastoconidia/arthroconidia (E). Panels F to L correspond to haploid sexual development: protoperithecia (F), paraphysal hyphae (G), and heterokaryotic ascogenous tissues (H). Panels I to L correspond to increasingly advanced sexual stages of meiosis and ascus development. The strain used as female in these experiments (H to L) contained *sms-4*$^+$ fused to a nonfunctional *gfp,* hence the absence of signal from the paraphysal hyphae.

distinguish between RNA coming from fertilized or unfertilized cells. Therefore, to observe *Sms-4* expression in different cell types during the Neurospora life-cycle, we compared the fluorescence of a C-terminal fusion of *gfp*$^+$ to *sms-4*$^+$ (*sms-4*$^+$::*gfp*$^+$) with that of strains containing a modified fusion with a stop codon inserted between the *sms-4*$^+$ and *gfp*$^+$ ORFs (*sms-4*+::*gfp**). Both alleles are functional in meiotic silencing (Crosses 29 and 62, data not shown and Figure 20).

During vegetative growth and development on solid media, SMS-4::GFP expression was detected: in mycelia, in structures developing asexual spores

(macroconidiophores), and in young asexual spores (blastoconidia) (Figure 19B to 3D). However, a *sms-4*$^{+}$::*gfp*$^{+}$-specific signal could not be detected in mature blastoconidia or arthroconidia (Figure 19E), either due to a lack of gene expression or an inability to detect GFP against the background of increased auto-fluorescence of these cell types or to a reduced penetration of the cell by excitation or emission light. SMS-4::GFP was also detected in haploid female tissues such as protoperithecia prior to fertilization and maternal tissues (paraphysal hyphae) following fertilization (Figure 19F and 3G, Crosses 25 and 26).

To observe *Sms-4* expression during meiosis, the GFP signal in crosses where the male contained the *sms-4*$^{+}$::*gfp*$^{+}$ fusion and the female contained *sms-4*$^{+}$::*gfp** mutant allele was monitored (Crosses 27 to 29). This procedure ensures that the GFP signal came only from the fertilized tissue and that the *sms-4*$^{+}$::*gfp*$^{+}$ allele had a pairing partner in meiosis. SMS-4::GFP was detected in the heterokaryotic ascogenous hyphae immersed in maternal tissue a few days post-fertilization (dpf) and was present throughout Meiosis I, Meiosis II and the post-meiotic mitosis, with the exception of each metaphase (Figure 19H to 3L, data not shown). As with mature conidia, SMS-4::GFP was not detected in mature ascospores, either because of melanization of the cell walls or because SMS-4 is not normally expressed in those tissues (data not shown). In summary, SMS-4 appears to be more or less ubiquitously expressed during at least the active stages of the Neurospora life cycle.

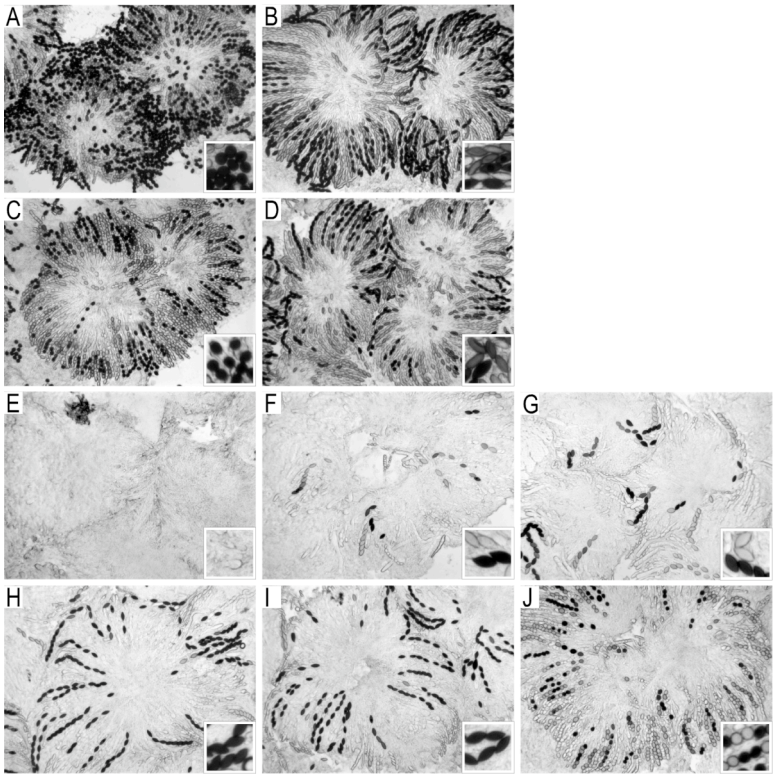

Figure 20. Testing the functionality of fusion proteins used in this study. Representative images from crosses given in Table 4 are shown. Images were captured at 63X. Insets at the lower right of each image are a 3X magnification of a region within the larger image and show ascospore morphology. A) The *sms-4*$^{+}$::*gfp*$^{+}$ fusion efficiently silenced unpaired *Rsp*. B) The function of this fusion was recessive to *Sms-4*^{UV}. C) The *sad-1*$^{+}$::*gfp*$^{+}$ fusion efficiently silenced unpaired *Rsp* and produced normal levels of ascospores. D) The silencing in the presence of *sad-1*$^{+}$::*gfp*$^{+}$ was still *Sms-4*-dependent. E) Perithecial tissues from *Sad-2* mutants most frequently lacked any ascospores. F&G) The presence of a single copy of *rfp*$^{+}$::*sad-2* increased the frequency of asci containing ascospores. H) The presence of two copies of *rfp*$^{+}$::*sad-2* did not increase the fertility anywhere close to wild-type levels. I) Despite partial complementation of the sexual defects of *Sad-2* mutation, *rfp*$^{+}$::*sad-2* was unable to silence unpaired *Rsp* in the few asci that developed ascospores. J) Round spores were produced when the same strain was crossed to a *Rsp* mutant. Despite the presence of a single functional copy of *sad-2*$^{+}$, the number of ascospore-producing asci was considerably higher than any of the *rfp*$^{+}$::*sad-2* complemented crosses.

Sms-4 is required for the meiotic silencing of all characterized classes of unpaired DNA

To determine how SMS-4 participates in meiotic silencing, we examined different types of meiotic silencing and the meiotic silencing resulting from different classes of inducers in *Sms-4* mutants. Thus far, there are two general classes of inducers of meiotic silencing. The first class consists of indel alleles (i.e., insertions or deletions). Deletions of DNA result in the unpairing of the equivalent region on the homologous chromosome. Similarly, insertions would be recognized as unpaired in meiosis, because the inserted regions lack homology on the opposite chromosome. Indels will be represented by alleles *Rsp*Δ, *Asm-1*^{ect}, *mat-A*$^{(IL->VR)}$, *hH3hH4-1*^{ect} and *hH1*$^{+}$*::gfp*$^{+}$. The second class consists of homeologous alleles, in which there is only partial homology at the allelic position of the homologous chromosome. This class is represented by alleles generated by RIP mutagenesis, *Rsp*^{RIP93} and *Rsp*^{RIP103}, whose inductive abilities are partially dependent on DNA methylation (Pratt *et al.* 2004).

Both indels and homeologous regions can induce the two types of meiotic silencing: *cis*- and *trans*-meiotic silencing. *Cis*-silencing is operates on the unpaired DNA itself, whereas *trans*-silencing must operate on paired DNA and is induced by an unpaired homologous region elsewhere in the genome.

Control crosses demonstrated that *Sms-4* loss-of-function alleles had no effect on spore morphology or color (Figure 21A, Crosses 30 and 45). We then tested the requirement for *Sms-4* in the *cis*-silencing induced by homeologous and indel alleles. The *Rsp*^{RIP103} allele readily induced *cis*-meiotic silencing in heterozygous crosses (*rsp*$^{+}$/*Rsp*^{RIP103}), as evidenced by the low percentage of spindle-shaped ascospores produced (Figure 21A, Cross 32). Unpairing *sms-4*$^{+}$ in this background reduced silencing, as evidenced by the ~10-fold increase in

the number of spindle-shaped ascospores (Figure 21A, Cross 33). When the same crosses were performed in the demethylated condition, the silencing induced by the *Rsp*^{RIP103} allele was weakened both when *Sms-4* was paired or unpaired (Figure 21A, Crosses 34 and 35, compare cross 32 to 34 and cross 33 to 35). As predicted, in crosses homozygous for *Sms-4*$^{\Delta}$ all meiotic silencing induced by *Rsp*^{RIP103} was lost, even when the allele was methylated (Figure 21A, Cross 36), a behavior that was also observed with the *Rsp*^{RIP93} allele (Figure 21A, Crosses 7 and 9).

The requirement for *Sms-4* in the *cis*-silencing induced by indels was tested using three inducers: *hH1*$^{+}$::*gfp*$^{+}$, *Rsp*$^{\Delta}$ and *mat-A*$^{(IL \rightarrow VR)}$. The *rsp*$^{+}$ allele was efficiently silenced in *rsp*$^{+}$/*Rsp*$^{\Delta}$ heterozygous crosses (Figure 21A, Cross 37), but no silencing was observed when the same crosses were performed either in an *Sms-4*$^{\Delta}$ or *Sms-4*^{UV} homozygous condition (Figure 21A, Crosses 38 and 39, respectively). The requirement for *Sms-4* in the *cis*-silencing induced by an ectopic insertion (*hH1*$^{+}$::*gfp*$^{+}$) was shown above (Figure 21, 4A to 4F, Cross 3). Mating type idiomorphs are particularly interesting as they are normally unpaired in meiosis, where they appear to be immune to silencing (Shiu *et al.* 2001). Ectopic translocation of *mat-A* (*mat-A*$^{(IL > VR)}$) resulted in reduced fertility (Figure 21B, Cross 40). This silencing was suppressed in either *Sad-1* or *Sms-4* heterozygous condition (Figure 21B, Crosses 41 and 42).

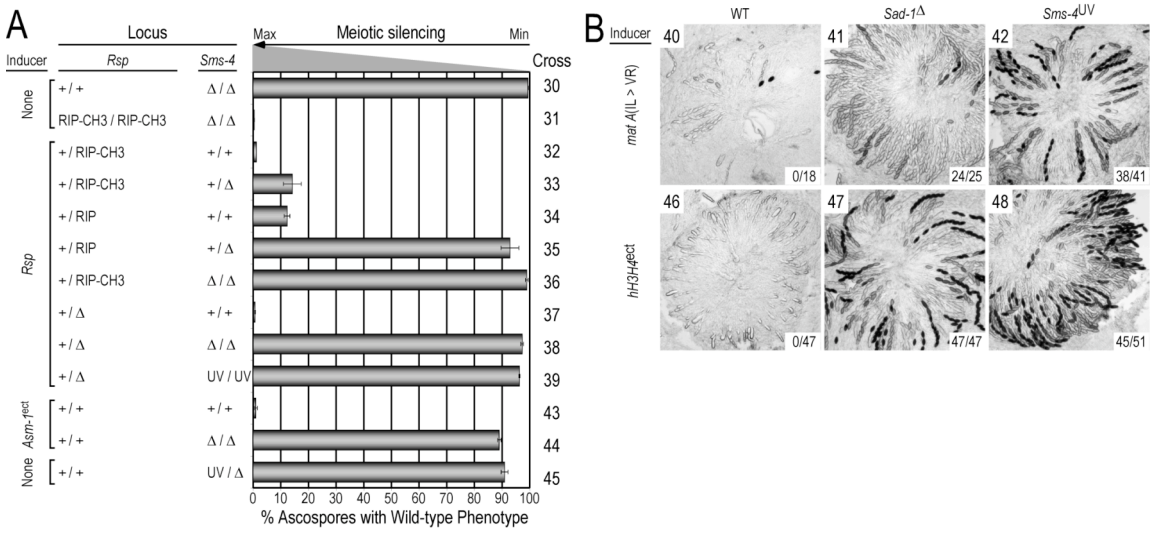

Figure 21. *Sms-4* is required for both classes of inducers of meiotic silencing and for *cis*- and *trans*-silencing. A) Quantification of meiotic silencing using inducers with an ascospore phenotype. The requirement for *Sms-4* in *cis*- and *trans*-silencing induced by homeologous and indel alleles was determined by quantifying the ratio of wild-type to mutant ascospores in each cross. Efficient meiotic silencing results in the production of ascospores that are round instead of spindle-shaped for *Rsp* (*cis*) and white instead of black for *Asm-1* (*trans*). The weaker the silencing the higher the percentage of wild-type ascospores produced in the cross. See text for details. B) Quantification of meiotic silencing using inducers with an ascus phenotype. The ectopic insertion alleles, *mat-A*$^{(IL > VR)}$ (upper row) and *hH3hH4-1*^{ect} (lower row), were used to test the requirement for *Sms-4* in meiotic silencing. Silencing by each inducer was tested in crosses to wild-type (WT), *Sad-1*$^{\Delta}$, and *Sms-4*^{UV}, as indicated above each column. The cross number is given on the upper left, and the ratio of wild-type to total rosettes analyzed is given on the lower right part of each image. Images were captured at 63X magnification. Weaker silencing results in the production of more ascospores.

Having shown that *Sms-4* is required for *cis*-silencing as induced by homeologous and indel alleles, its requirement in *trans*-silencing was then tested using two ectopic inducers: *Asm-1* and *hH3*::*hH4-1*. The ectopic insertion of the *Asm-1* gene (*Asm-1*^{ect}) induced silencing of its paired alleles at the canonical position (Figure 21A, Cross 43). In the *Sms-4*$^{\Delta}$ homozygous background, there was no meiotic silencing observed (Figure 21A, Cross 44). Similarly, the ectopic insertion of the *hH3hH4-1* genes (*hH3hH4-1*^{ect}) induced silencing of the paired alleles at the canonical position (Figure 21B, Cross 46), resulting in ascus arrest. Unpairing of *Sad-1* or *Sms-4* in these crosses reduced silencing (Figure 21B, Crosses 47 and 48).

Together these crosses demonstrate that deletion of *Sms-4* is dominant in meiosis and that *Sms-4* is required for both *cis*- and *trans*-silencing and silencing induced by both homeologous and indel alleles.

Sms-4 is not required for quelling induced by a hairpin RNA

If *Sms-4* encodes a conserved component of RNAi, then either it, or a paralog, should function in quelling. *Sms-4* lacks a paralog, so its expression in vegetative cells is consistent with a possible role in quelling. We assayed quelling induced by a hairpin RNA directed against the carotenoid biosynthesis gene *albino-1*$^{+}$ (*al-1*$^{+}$) (Figure 22). In wild-type strains expressing the hairpin, Neurospora mycelia had a white or yellow color instead of the wild-type orange color. When the hairpin was present, *qde-2* mutants failed to quell *al-1*$^{+}$, as previously shown (Goldoni *et al.* 2004). While reportedly required for quelling of repeated transgenes and endogenous RIPped repeated elements in the genome (Cogoni and Macino 1999; Nolan *et al.* 2005), *qde-1* is not required for silencing hairpin RNAs or TAD transposable elements (Cogoni and Macino 1999; Nolan *et al.* 2005). *Sms-4* mutants silenced the *al-1*$^{+}$ gene efficiently when the hairpin was present; demonstrating that *Sms-4* is not required for quelling induced by hairpin RNAs. We cannot rule out a role for *Sms-4* in the earlier steps of quelling because the detection of *qde-1*-dependent quelling has unfortunately remained elusive in our lab.

SMS-4 is a nuclear protein whose localization is independent of known components of meiotic silencing

The subcellular localization of a protein can hint at its possible roles. We therefore determined the subcellular localization of the SMS-4::GFP fusion during meiosis. As subcellular control markers, we used a perinuclearly localized RFP::SAD-2 fusion protein (Shiu *et al.* 2006) and Hoechst stained DNA. SMS-

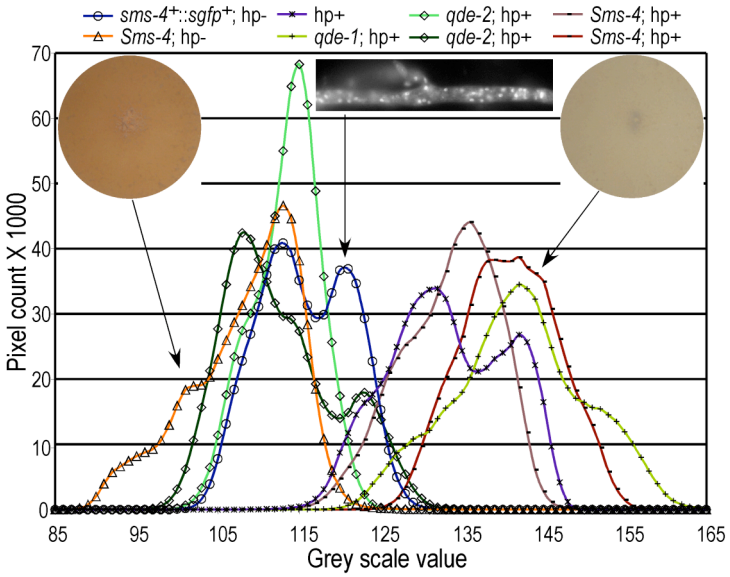

Figure 22. *Sms-4* is not required for the quelling of a hairpin RNA. Strains *sms-4*$^{+}$::*gfp*$^{+}$ hp^{-} (RPNCR506A), *Sms-4*, hp^{-} (RPNCR333A), hp^{+} (RPNCT140A), *qde-1*, hp^{+} (RPNCT189A), *qde-2*, hp^{+} (TGNCT1A and TGNCT1B) and *Sms-4*, hp^{+} (RPNCT567A and RPNCT567B) were grown on Petri plates and photographed. Grey scale histograms were obtained, which plot the pixel counts (y-axis) for each grey scale value (x-axis). Strains with a wild-type orange phenotype (left inset) were darker resulting in lower gray-scale values whereas strains that silenced *al-1*$^{+}$ were yellow-white and lighter (right inset) resulting in higher gray-scale values. SMS-4::GFP was detected under these growth conditions (center inset).

4::GFP fluorescence appeared as a diffused cloud enveloping the DNA within the nucleus showing little or no overlap with the perinuclear RFP::SAD-2 protein (Figure 23A, Cross 49). Also, SMS-4::GFP co-localized with nuclear DNA in all tissues in which the fusion was detected (data not shown).

To determine if localization of SMS-4::GFP to the nucleus during meiosis was dependent on other known components of meiotic silencing, we looked at SMS-4::GFP localization relative to Hoechst stained chromatin in *Sad-1*, *Sad-2*, *Sms-2* and *Sms-3* mutants (Figure 23B, Crosses 50 to 54). None of these mutations affected SMS-4 localization to the nucleus despite the absence of or reduction in meiotic silencing. It should be noted, however, that since crosses homozygous for *Sms-2* and *Sms-3* fail to produce asci, these crosses had to be heterozygous

for dominant mutant alleles (Crosses 53 and 54). Given that the observed dominance was likely due to meiotic silencing, the gene products for *Sms-2* and *Sms-3* were most likely needed for silencing their own transcripts at least at some point during the cross. Therefore it is possible that this residual *Sms-2* and *Sms-3* activity functioned in SMS-4 localization during meiosis in these mutants. *Sad-1* and *Sad-2* crosses (Crosses 51 and 52) were homozygous for the mutation and did not have this complication, however, they were complicated by their meiotic arrest.

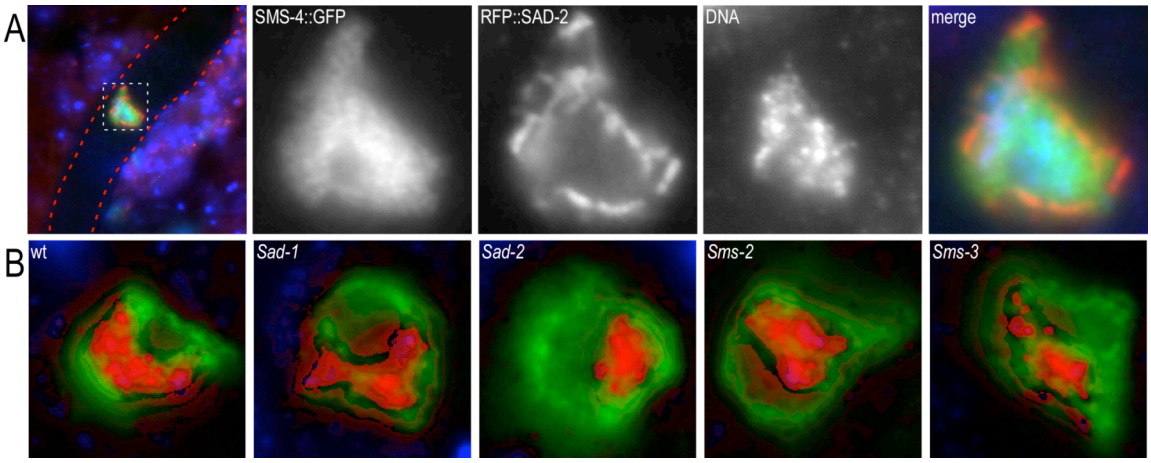

Figure 23. SMS-4 is a nuclear protein. A) Localization of SMS-4 relative to nuclear DNA and the perinuclear SAD-2. Upper panel images are from a Prophase I ascus expressing SMS-4::GFP (green), RFP::SAD-2 (red) and DNA stained with dye Hoechst 33258 (blue). The ascus is outlined in red in the first merged image of the panel. The next four adjacent images were all derived from the white dotted boxed region surrounding the nucleus in this first image. B) Localization of SMS-4::GFP relative to DNA in wild-type and *Sad-1, Sad-2, Sms-2* and *Sms-3* mutants. In these images, red represents the overlap between the SMS-4::GFP and DNA (Hoechst) signal within Prophase I nuclei. Green is where SMS-4::GFP signal was greater than the DNA signal and blue is where the DNA (Hoechst) signal was greater than SMS-4::GFP signal. Images were generated by the merging of three images that were each obtained by: 1) subtracting GFP from DNA signal, 2) subtracting DNA from GFP signal and 3) calculating the intersection of GFP and DNA signals.

Localization of Sad-1 to the perinuclear region is independent of *Sms-4*

It has been previously reported that the localization of an ectopically-inserted, overexpressed SAD-1::GFP fusion is dependent upon *Sad-2* (Shiu *et al.* 2006). We therefore hypothesized that *Sms-4* might have a similar role in meiotic silencing. To test this, we determined SAD-1 localization in *Sms-4* mutants. A fusion of *sad-1*$^+$ to *gfp*$^+$ (i.e., *sad-1*$^+$::*gfp*$^+$) was inserted under the control of its own promoter at the *Sad-1* chromosomal position. Homozygous crosses of strains carrying this fusion produced normal rosettes and silenced *rsp*$^+$ efficiently, demonstrating the functionality of the fusion in both sexual development and meiotic silencing. Furthermore, the silencing of *Rsp* driven by this fusion protein was *Sms-4*-dependent (Figure 20, Crosses 64 and 65).

Localization of the sad-1::gfp fusion was therefore analyzed in a wild-type background, and in *Sad-2*, *mei-3* and *Sms-4* mutant backgrounds (Figure 24, Crosses 55 to 59). In wild-type, sad-1::gfp could be detected in nearly all ascogenous tissues as cytoplasmic foci and was notably absent from paraphyses despite the fusion allele being present in both the male and the female. In addition to a few cytoplasmic foci, it also localized around the nucleus in developing meiotic asci and persisted there until sometime shortly prior to the first meiotic division, at which time it dispersed to cytoplasmic foci within the ascus for the remainder of ascogenesis (Figure 24A, Cross 55 and data not shown).

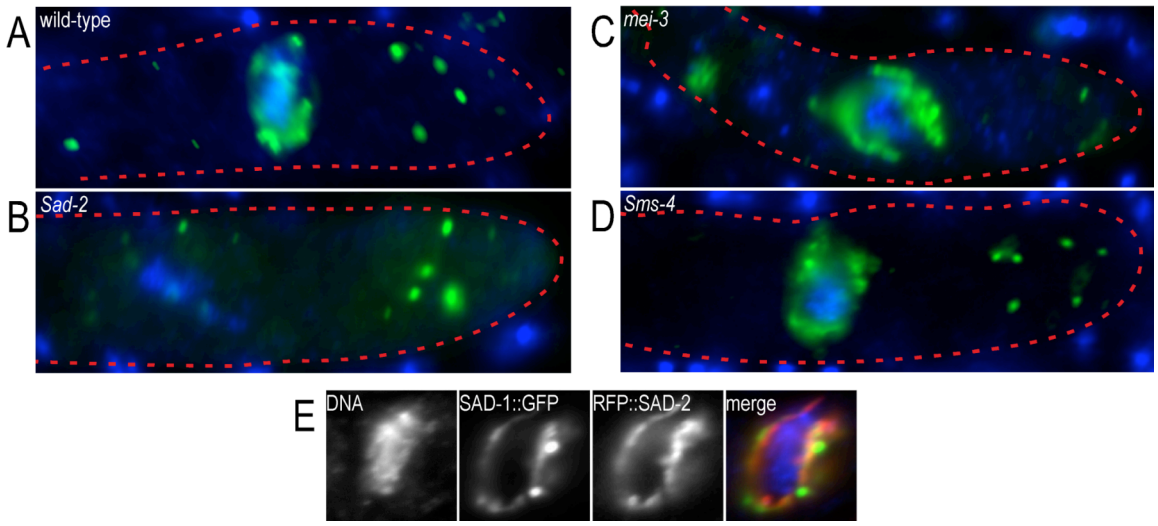

Figure 24. Normal SAD-1::GFP perinuclear localization is *Sad-2*-dependent but independent of *mei-3* and *Sms-4*. A-D) SAD-1::GFP localization in wild-type (A) and *Sad-2* (B), *mei-3* (C) and *Sms-4* (D) mutants in Prophase I asci. Dotted red lines outline asci. Green and blue represent SAD-1::GFP and DNA (Hoechst), respectively. E) Localization of SAD-1 and SAD-2 around the nucleus during meiotic Prophase I is independent of the presence of SMS-4. In the merged image SAD-1::GFP is green, RFP::SAD-2 is red and Hoechst stained chromatin is blue.

As expected, normal perinuclear localization of sad-1::gfp fusion was affected in a *Sad-2* mutant. In theory, the affected localization in the *Sad-2* mutant could be an indirect consequence of meiotic arrest. However, despite displaying a meiotic arrest phenotype similar to that of the *Sad-2* mutant (Raju and Perkins 1978), a *mei-3* mutant exhibited sad-1::gfp perinuclear localization (Figure 24B and 24C, Cross 56 and 57, respectively). In the *Sms-4* mutant, sad-1::gfp remained perinuclear (Figure 24D, Cross 58) and had a similar pattern as RFP::SAD-2 (Figure 24E, Cross 59), demonstrating that the role of SMS-4 in meiotic silencing is not related to controlling localization or expression of *Sad-1*.

DISCUSSION

In summery, we have identified a novel conserved gene required for meiotic silencing by unpaired DNA in *Neurospora crassa*, *Sms-4*. This characterization of *Sms-4* has shed light on the process of meiotic silencing. First, it is clear that

meiotic silencing by unpaired DNA is not absolutely required for meiosis, contrary to the suggestion by the barren-phenotype of loss of function mutants of all previous components. Importantly Sms-4 is the first component of meiotic silencing to localize with bulk chromatin in the nucleus, presumably the location of unpaired DNA, confirming that nuclear events are required for meiotic silencing. *Sms-4* provides the first genetic evidence for the function of the conserved class of proteins to which it belongs, other members of which have been recently isolated as part of the mRNP in humans. Furthermore, bioinformatic analysis predicted that these proteins contain an RNA recognition motif and thus may bind RNA directly.

What is the role of *Sms-4* in meiotic silencing?

At this point we can only speculate on the role of *Sms-4*. Where could an RNA-binding nuclear protein function in the modeled meiotic silencing pathway (Kelly and Aramayo 2007)? Meiotic *trans*-sensing is inherently nuclear, since the homologous chromosomal loci that are compared are only present in the nucleus (Aramayo and Metzenberg 1996). But why should an RNA-binding protein be involved? It is clear that *trans*-sensing occurs independent of synapsis and is likely to be independent of stable homologue alignment and most meiotic recombination (Chapter III). It is possible that unstable recombination-independent homologue pairing occurs between DNA molecules (Gerton and Hawley 2005; McKee 2004; Zickler 2006). However, it is also possible that *trans*-sensing between homologous chromosomal loci could be mediated by RNAs stationed at these loci. Such a mechanism could allow for base-pair interactions without the need to disrupt continuity of the DNA strands. *Sms-4* could be a critical component of this hypothetical RNA-based *trans*-sensing. Clearly this *trans*-sensing would be dispensable for chromosome pairing, since *Sms-4* mutants are meiotically normal (Figure 16).

Interestingly, the presence of SMS-4 orthologs in Fungal/Metazoan lineages correlates with a requirement for double-strand breaks (DSBs) for chromosome pairing in meiosis. Perhaps SMS-4 participates in active chromosome *trans*-sensing early in meiosis during DSB-independent homolog recognition. Indeed, the activity of this SMS-4-mediated *trans*-sensing could be responsible for the instability that is observed in early chromosome pairing. Flies and worms lacking an Sms-4 ortholog synapse their homologues using DSB-independent mechanisms in meiosis, whereas Neurospora and mice, which contain an Sms-4 ortholog, require DSBs for stable homologue synapsis (Bowring *et al.* 2006; Dernburg *et al.* 1998; McKim and Hayashi-Hagihara 1998; Romanienko and Camerini-Otero 2000). In agreement with this idea, SMS-4 is absent from *S. cerevisiae* and *Coprinus cinereus,* which still exhibit significant pre-synaptic homologue pairing in the absence of DSBs (Celerin *et al.* 2000; Cha *et al.* 2000). One exception to this idea is *S. pombe*, which contains an SMS-4-like protein but still manages to pair its chromosomes in the absence of DSBs (Nabeshima *et al.* 2001). Perhaps, in *S. pombe,* the horsetail movements of the nucleus between cell poles facilitate DSB-independent homologue pairing (Yamamoto and Hiraoka 2001) overriding or following SMS-4-mediated processes. It should be noted that the pre-synaptic homologue pairing in the absence of DSBs has not been as closely scrutinized in Neurospora as it has in some other organisms, although it has been well characterized in *Sordaria macrospora*, a member of the same taxonomic family (Storlazzi *et al.* 2003; Tesse *et al.* 2003). This model predicts stable homologue pairing in *Sms-4* mutants defective in DSB formation in Neurospora.

Another attractive possibility is that SMS-4 acts as a conduit between meiotic *trans*-sensing and meiotic silencing. Since meiotic silencing is likely an RNA-based mechanism, it is presumed that some sort of aberrant RNA (aRNA) molecule is transcribed from the unpaired DNA, which then serves as the

initiating substrate for RNA silencing (Kelly and Aramayo 2007). Since the unpaired DNA is in the nucleus and the RNAi machinery is perinuclear and cytoplasmic, the efficiency of silencing might be increased by the presence of a chaperone protein that transports the aRNA to the silencing apparatus. Perhaps, SMS-4 or a modified form of it, defines aRNA. For instance, it is conceivable that SMS-4 binds to RNA at the chromosomal locus shortly after transcription, perhaps as part of the mRNA processing machinery. If this RNA is in an unpaired region identified by meiotic *trans*-sensing, then SMS-4 might be post-translationally modified. These mRNAs would then have a one-way ticket to the silencing apparatus at the nuclear periphery or in the cytoplasm.

This role for SMS-4 would parallel the proposed function of kinesin KIF17b in chromatoid biology during post-meiotic mouse spermatogenesis (Kotaja *et al.* 2006; Kotaja and Sassone-Corsi 2007). KIF17b has been proposed to carry packages of protein-bound mRNAs from the nucleus to the chromatoid body that, like the meiotic RNAi machinery in Neurospora, is located outside along the nuclear periphery. The chromatoid body contains many RNAi-associated proteins (Kotaja and Sassone-Corsi 2007). Interestingly, one of these proteins, Maelstrom (MAEL), localizes to unsynapsed chromatin during mouse meiosis and interacts with the mouse Vasa Homologue protein (MVH), which also localizes to the chromatoid body and interacts with RNAi machinery (Costa *et al.* 2006). If ELG proteins could are critical for transport of aRNA from the nucleus to the chromatoid body, such activity could explain the unexplained genetic behavior of the mouse paramutable Kit gene (Rassoulzadegan *et al.* 2006). In that case the aRNA produced by the unpaired DNA may be transported to the chromatoid body, where it would be stored to silence homologous regions in the next generation.

The effect of *Sms-4* mutation on meiotic silencing might also be indirect. For example, since the predicted secondary structure of Region A is similar to the RNA recognition motifs of two RNA-binding proteins required for alternative splicing (Black 2003), SMS-4 may be required for the efficient splicing of a component of meiotic silencing. In the absence of SMS-4, the amount of protein from this hypothetical gene could drop below that required for meiotic silencing while, maintaining sufficient amounts for a normal meiotic progression. Unfortunately, as is the case for all mutants that have been identified by genetic analysis of meiotic silencing, including *Sad-1,* it is difficult to know if the requirement for any of the genes involved in meiotic silencing is direct or indirect.

What is the role of RNAi in meiosis?

The apparent disconnect between meiotic silencing and meiosis itself demonstrated by the *Sms-4* mutants raises a key question: What is the role of meiotic silencing in meiosis? Based on developmental defects, the known mutants of meiotic silencing can be separated into at least three classes: mutants that fail to accumulate ascogenous tissue and are blocked very early in sexual development (e.g., *Sms-2* and *Sms-3*) (Alexander *et al.* 2007; Lee *et al.* 2003b), mutants that fail to progress beyond Meiosis I (e.g., *Sad-1* and *Sad-2*) (Shiu *et al.* 2006; Shiu *et al.* 2001), and mutants with normal meiosis (e.g., *Sms-4*). If meiotic silencing by unpaired DNA is not required for meiosis, the meiotic defects of *Sad-1*, *Sad-2*, *Sms-2* and *Sms-3* mutants are probably due to other biological roles of these genes. The shared perinuclear localization of SAD-1, SAD-2, SMS-2 and SMS-3 (Alexander *et al.* 2007; Shiu *et al.* 2006), is consistent with these proteins having a role at the same developmental stage, although the earlier arrest of *Sms-2* and *Sms-3* mutants suggests an additional role of these in early sexual development. The participation of SMS-4 in meiotic silencing is in agreement with the predicted involvement of these proteins in a

yet-to-be defined part of meiotic RNA metabolism, probably through its potential RNA recognition motif.

If we assume that the sole function of the previously identified components is RNA silencing, then they must be silencing something important, since mutants like *Sad-1* and *Sad-2* are blocked in development. The necessary substrate of SAD-1 and SAD-2 is either derived from unpaired DNA or something else. Given that we have shown that *Sms-4* is required for silencing many types of unpaired DNA (*viz*., *asm-1*$^{+}$, *hH1*$^{+}$::*gfp*$^{+}$, *hH3hH4-1*$^{+}$, *mat-A*$^{+}$*, rfp*$^{+}$::*sad-2*, *rsp*$^{+}$, *sad-1*$^{+}$::*gfp*$^{+}$, *Bml*$^{+}$::*gfp*$^{+}$, *mei-2*$^{+}$, *msh4*$^{+}$*,* and *spo76*$^{+}$::*gfp*$^{+}$), we think that the necessary substrate of SAD-1 and SAD-2 for development is something else.

It is possible that in meiosis some paired loci directly trigger meiotic silencing by producing dsRNA from convergent promoters or some other form of aberrant RNA. The components of the meiotic silencing machinery required for development, e.g., SAD-1, would be required to silence all triggers, whereas other components not required for development, e.g., SMS-4, would be required only for processing of unpaired DNA. This role for *Sms-4* would be consistent with a role in *trans*-sensing or the transport of aRNA from unpaired DNA to the silencing machinery. If *Sms-4* is involved in quelling, an upstream role in silencing could also explain why *Sms-4* is not required for the quelling induced by a hairpin RNA.

Biochemical analysis of the mammalian ELG protein raises interesting questions about the role of *Sms-4* for RNA silencing. Mouse ELG and Neurospora Sms-4 are 21% identical across roughly 70% of their protein sequences. Human ELG belongs to a complex of proteins that bind processed mRNA in HeLa cells. Its presence in the mRNP complex depends on splicing and capping of the message (Merz *et al.* 2007). Thus, it is possible that SMS-4 is used only for

meiotic silencing of capped, polyadenylated, and/or spliced messages. If so, then RNA transcribed by RNA polymerase I or III might not be silenced in an SMS-4-dependent manner.

In conclusion, this analysis of *Sms-4* has defined a new class of gene products required for meiotic silencing by unpaired DNA. It has also sheds some light on the possible roles of the conserved family of ELG proteins. Through biochemical and further genetic analysis of meiotic silencing we hope to gain insight into mechanisms for controlling fertility and genome defense.

MATERIALS AND METHODS

Strain description and manipulation

All *N. crassa* strains used in this study are described in Table A1. The formulas for the Vogel's Medium N, the Westergaard's Medium, and the sugar mixture of Brockman and de Serres have been described by Davis and de Serres (1970). *Escherichia coli* K12 XL1-Blue MR (Stratagene) was the host for all bacterial manipulations. Growth conditions, conidial spheroplast preparation and fungal transformation were performed as described (Pratt and Aramayo 2002). Homokaryon purification was performed as described (Lee *et al.* 2003a; Pratt and Aramayo 2002). All cosmids used in this study were from the Orbach/Sachs pMOcosX library (Orbach and Sachs 1991) obtained from the FGSC (McCluskey 2003).

Quantification of *Rsp* silencing was as described (Pratt *et al.* 2004). For quantification of *Asm-1* silencing, progeny from directional crosses were allowed to shoot onto Petri dish lids that had been overlaid with 2% agar + 3 mM EDTA to prevent conidia from germinating and consuming the white ascospores. Pictures of ascospores were taken directly from the lid of the Petri dish 18 days post-fertilization (dpf) and the spores quantified. Neurospora genetics,

dissection of perithecia, and preparation of sexual tissues from crosses were as described (Chapter III) except when stained with Hoechst, rosettes were transferred to 90 µl STC + 5 µg/ml Hoechst following removal from the perithecia. When fixing tissues for analysis, a group of perithecia were removed from the plate with a scalpel then transferred to a tube containing 1 ml of fixative solution (10mM Pipes, pH6.9, 10mM EGTA, 10mM magnesium sulfate, 0.3% Triton X-100, 3.7% formaldehyde) and incubated at room temperature for 30 min. Perithecia were then washed twice in several milliliters PBS, transferred to a 200µl tube containing 100µl of STP and then prepared as described above for unfixed tissues.

SMS-4::GFP expression analysis

Strains RPNCR506A and RPNCR500A were point inoculated onto the center of Petri dishes containing supplemented Westergaard's media (1.5% sucrose) and incubated at room temperature. Mycelia, macroconidiophores and young blastoconidia were plucked from the plates 3 days post-inoculation (dpi) and transferred to a pool of STC + 5 µg/ml Hoechst 33258 or 1.5 µg/ml DAPI. Older blastoconidia and protoperithecia were similarly processed 6 dpi. At this time, the culture was fertilized (crosses 25 & 26) and paraphyses were extracted from perithecia at 3 dpf. Perithecial contents from crosses 27-29 were handled at 3 to 7 dpf as described above. Fluorescence images were captures at 600X magnification using a Photometrics Cool Snap HQ^2 set to autoexposure. *Gfp*$^+$ and *gfp** cultures were analyzed in parallel.

Assay of quelling of *albino-1*

Petri dishes containing supplemented Westergaard's media (1.5% sucrose) were point inoculated at the center from fresh 1 ml starter cultures and incubated at 24 C for 3 days under constant light. Plates were then transferred to 6 C for 3

days to allow for carotenoid accumulation. Two pictures were then taken for each strain using a Kodak DC290 digital camera. Using the program ImageJ (NIH), a centered circle of a 900 pixel diameter covering most of the plate was selected and used to calculate a histogram. Grey scale values were calculated from RGB images using unweighted red, green and blue values. Histogram data from the pictures was pasted into Microsoft Excel where the values of the two pictures of each strain were averaged. The detection of SMS-4::GFP in RPNCR506A confirmed expression under these culturing conditions.

Molecular biology

Procedures for cloning, DNA analysis, sequencing, Southern blot analysis and other nucleic acid manipulations were performed as described (Pratt and Aramayo 2002). Oligonucleotides or primers used in this study are listed in Table A2. To determine the transcriptional start and poly(A) addition sites of *Sms-4*, 5' and 3' RACE was performed using the BD SMART RACE cDNA Amplification Kit as directed by the manufacturer protocol using total RNA extracted from RANCR05A grown in supplemented Westergaard's liquid media. Gene-specific primers ORP157 and ORP156 were used for 5' and 3' amplification respectively. The open reading frame of *Sms-4* was amplified from the same cDNA using ORP170 and ORP171. Cloned PCR products were sequenced.

Northern blot analysis

All cultures were inoculated in parallel with an equivalent-sized plug from either RANCR49A (*sms-4*$^{+}$) or RPNCR321A (*Sms-4*Δ) grown on supplemented solid Vogel's media. For liquid media, plugs were inoculated into 30 ml supplemented liquid Vogel's or Westergaard's media (1.5% sucrose) in 125 ml flasks. Cultures were incubated in a room-temperature water bath with constant mixing and constant light for 6 days prior to RNA extraction (Figure 19A, lanes 1-4). For

solid media, plugs were inoculated onto solid supplemented Vogel's or Westergaard's media (1.5% sucrose) overlaid with a washed cellophane circle. Cultures were incubated for 6 days at room-temperature in constant light. RNA was extracted from some of these cultures (Figure 19A, lanes 5-8). Others were fertilized as outlined in Table 4, crosses 21-24 (Figure 19A, lanes 11-14) by applying conidia of the male suspended in media or mock-fertilized with un-inoculated media (Figure 19A, lanes 9 & 10). These were incubated at room temperature for an additional 6 days before extracting RNA.

To prepare the cellophane, it was cut into uniform plate-sized circle, placed into 1 L of deionized water and cooked in the microwave for 25 mins. They were washed two more times by replacing the water with fresh water then cooking again. The washed cellophane circles were sandwiched between Kim-wipes, stacked, wrapped in aluminum foil then autoclaved. The evening before inoculation, cellophane circles were hydrated in sterile water for a couple minutes, laid on top of solid media in a Petri dish, then left at room-temperature. The cellophane facilitated harvesting the tissue, which could simply be peeled of the surface of the cellophane.

To extract total RNA, cultures from liquid were drained of media and dried on paper towels then immediately transferred to liquid nitrogen, ground to a powder then transferred to 2.5 ml Trizol reagent in a 50 ml conical tube. Cultures from solid media were peeled off the cellophane, rolled up then transferred to liquid nitrogen, ground to a powder and transferred to 2.5 ml Trizol reagent in a 50 ml conical tube. One plate or flask supplied sufficient tissue for the extraction for each condition however different cultures for these conditions were analyzed. RNA was extracted as protocoled by the Trizol manufacturer. To further clean the RNA, this was followed by a lithium chloride precipitation, three chloroform extractions and a sodium acetate (pH 4.8)/isopropanol precipitation. RNA from

solid media, particularly the older cultures, needed to be cleaned to get more reliable quantifications by spectrometry. The Northern blot was performed using standard procedures with 20 µg of total RNA loaded per sample. The probe for *sms-4*$^{+}$ was the 2.2-kbp *Pst*I-*Spe*I fragment from its locus and the probe for *spo11*$^{+}$ was the 1.2-kbp *Eco*RI-*Xba*I fragment from its locus.

Mutagenesis

A 10 ml conidia suspension from KYNCT13A in water was irradiated with UV then immediately applied to a female of RANCR49A. Spores were collected then germinated using standard techniques in the presence of a sugar mixture that restricts radial growth. Colonies were transferred from the agar plates to 1 ml liquid culture tubes and incubated at 35 C, then at 25 C to stimulate conidiation. We discarded the approximately 50% of the viable progeny that were *fl*p and did not conidiate. The remaining progeny were individually spotted onto KBNCR5A and KBNCR6A females to test for suppression of *Rsp*^{RIP93} dominance, as well as KBNCR1A and KBNCR2A, DLNCR142 and DLNCR147, and MMNCR01A and MMNCR11A females to test for complementation of *Sad-1*, *Sms-2* and *Sms-3* sterility. Strains producing ascospores in these crosses were then crossed to RANCR49A or RANCR50A depending on the mating-type of the original isolate. Progeny from these were scored for their ability to suppress the dominance of *Rsp*^{RIP93} and analyzed by Southern blot to ensure that they lacked the insertion of *Asm-1* at *his-3*. Two isolates, one of each mating-type, from each mutant was given the same numerical designation prefixed by the letter S (for suppressor), e.g., S1 A in Table A1. Following the characterization of the *Sms-4*^{UV} mutation in suppressor S1, Southern blotting determined that S2, S4 and S5 contained the same UV allele and were likely siblings. A *Sad-2*^{UV} allele was identified in mutant S10 by Southern blot.

pKYAM071, used to create strain KYNCT13A, was constructed by cloning the 2,080 bp *Eco*RI-*Bam*HI fragment (coordinates 4615 to 6690) from pKYAM029 (Kutil *et al.* 2003) into the *Eco*RI-*Bam*HI sites of pKYAM026 (Lee *et al.* 2003a).

Construction of alleles and mutant strains

The pRATT18 series: The root-vectors for our *hph*$^+$::*kan* selection marker and *gfp*$^+$ plasmids. For reasons not directly related to this work, we developed a Mu-derived transposon containing a fungal selection marker that could be used for *in vitro* insertional mutagenesis. For the purposes of this work, it contained the positive selection marker *hph*$^+$ in a relatively small vector with convenient restriction enzyme sites. First the GeneJumper-KanR mini-mu transposon was inserted into pRATT08c (Pratt and Aramayo 2002) by *in vitro* transposition as directed by the manufacturer of the GeneJumper Primer Insertion Kit (Invitrogen), yielding pRATT18a. To obtain pRATT18b, the transposable element was amplified from this plasmid by PCR using ORP031 and blunt-end cloned into the unmethylated *Stu*I site of pRATT08a, which was itself constructed as described for pRATT08c (Pratt and Aramayo 2002) except using pBluescript (Stratagene) as the PCR template. An *Eco*RI fragment containing the *hph*$^+$ selection marker from pCB1003 (Carroll *et al.* 1994) was inserted into the *Eco*RI site of this plasmid such that *hph*$^+$ and *npt*$^+$ code in the same strand, yielding pRATT18c. A *Not*I site was then removed by digestion, Klenow fill-in and self-ligation, yielding pRATT18d. When using the transposon in pRATT18c for *in vitro* transposition there was high background from the parental vector. To reduce this background we derived a cloning vector from pKD4 (Datsenko and Wanner 2000) containing the R6K origin of replication that relies on *trans*-acting *pir*$^+$ protein from the host *E. coli* for replication. This was accomplished by PCR amplification from pKD4 with ORP014 and ORP027 followed by *Cla*I digestion, Klenow fill-in and self-ligation, yielding pRATT17b. pRATT18e resulted by PCR

amplifying the transposable element in pRATT18d with ORP031 and subcloning it into the *Swa*I site of pRATT17b.

For those that care, the transposon does work for *in vitro* transposition with commercially available transposase, however, the original application we created it for never reached fruition.

Scanning mutagenesis of Sms-4 region. The plasmid used to delete NCU01309, was constructed by PCR amplifying the two homologous flanks for replacement from cosmid G23:G9 (left: ORP148 & ORP149; right: ORP150 & ORP151). *Bam*HI digested PCR products were then mixed and ligated. The ligation product of the left and right flanks was then amplified by PCR using the two outside primers (ORP148 and ORP151). This was digested *Pst*I and subcloned into the *Pst*I site of pDL96a (to be described elsewhere) containing the *mating-type a* gene for use as a counter selection marker during transformation (Pratt and Aramayo 2002) yielding pRATT78a. For positive selection, the *Bgl*II fragment from pRATT18e was inserted between the flanks at the *Bam*HI site yielding pRATT78. pRATT79, used to delete NCU01310/*Sms-4*, was constructed by the same basic strategy as pRATT78 using primers ORP150, ORP151, ORP152 and ORP153. The only exception being that flanks were digested *Nsi*I prior to inserting into the *Pst*I site of pDL96a. Also pRATT80, used to delete NCU01311, was constructed by the same basic strategy as pRATT78 using primers ORP152, ORP153, ORP154 and ORP155.

pRATT81 and pRATT83, used to RIP the large intergenic region, were constructed by subcloning the *Bgl*II fragments of cosmid G23:G9 ranging from ~2.0 – 3.5-kbp into the *Bam*HI site of *pan-2*-insertion plasmid pRATT42b (to be described elsewhere). pRATT81 contains the 2.1-kbp fragment referred to as

intergenic region-1 (*IG-1*) in Figure 18. pRATT83 contains two *Bgl*II fragments, the adjacent 2.4 and 3.3-kbp fragments referred to as *IG-2* in Figure 18.

Sms-4 complementation. pRATT100, containing the wild-type *sms-4*$^+$ locus, was constructed by subcloning the 7.0-kbp *Hind*III fragment from cosmid G23:G9 spanning *sms-4*$^+$ into the *Xba*I site of the *his-3*-insertion vector pJHAM003 (Haag and Aramayo 2003) using half-filled-in restriction sites. pRATT107, containing the *sms-4*W97A allele, was constructed by PCR amplifying two halves of the *sms-4* gene from pRATT100 using primers ORP177 with His3D and ORP178 with His3U. ORP177 and ORP178 were both kinased with T4 PNK prior to PCR. Nucleotides in the 5'-tails of ORP177 and ORP178 change the codon for tryptophan at amino acid position 97 to an alanine. The PCR products were digested with *Bam*HI and subcloned into the *Bam*HI site of *his-3*-insertion vector pJHAM004 (Haag and Aramayo 2003) by way of a three-fragment ligation. Similarly, pRATT107*, containing the *sms-4*W97* allele, was constructed as for pRATT107 except using ORP186 with His3D. Nucleotides in the 5'-tail of ORP186 and ORP178 change the codon for tryptophan at amino acid 97 to a stop and introduce a frame-shift mutation.

Construction of fusions to green fluorescent protein. The version of GFP used in these studies is *S65T green fluorescent protein* (*sgfp*) taken from pMF280 (Folco *et al.* 2003; Freitag *et al.* 2004a). For clarity, except for in the genotypes Table A1, it is simply referred to as *gfp*. The flanks and *gfp* regions of all plasmids were sequenced to verify that unintended mutations were not introduced by PCR.

pRATT121 contains the sequences for creating a fusion between *sporulaiton76* (*spo76*$^+$, NCU00424.3) and *gfp* (*spo76*$^+$::*gfp*$^+$) by homologous recombination at the *spo76* locus. Importantly for this work, it serves as the root-vector for similar

C-terminal *gfp*$^+$ fusions constructs for other loci by replacement of the *spo76* homologous flanks with those of other loci. The left flank for homologous targeting was amplified from cosmid G16:A9 with ORP204 and ORP205 then digested with *Bam*HI and *Spe*I. The right flank was similarly amplified with ORP206 and ORP207 then digested with *Bam*HI and *Hind*III. *Gfp*$^+$ was amplified from pMF280 (Folco *et al.* 2003) with OTL001 and ORP111 then digested with *Spe*I and *Pst*I. These were ligated via a four-fragment ligation to an *Nsi*I-*Hind*III digested fragment from pRATT18c containing the plasmid origin of replication and *bla* gene for propagation and selection in *E. coli* and the *hph*$^+$ gene for selection in Neurospora.

pRATT122, containing the *sms-4*$^+$::*gfp*$^+$ fusion was constructed by replacing the *spo76* flanks of pRATT121 with PCR amplified regions from pRATT107. The left flank for homologous targeting was amplified with ORP212 and ORP213 then digested with *Bam*HI and *Xba*I. The right was similarly amplified with ORP214 and ORP153 then digested with *Bam*HI and *Hind*III. These were then ligated to *Spe*I-*Hind*III digested pRATT121. To introduce a stop codon between *sms-4* and the *gfp*, creating allele *sms-4*$^+$::*gfp**, pRATT122 was PCR amplified with OTL001 and a T4 PNK kinased ORP171 then self-ligated yielding pRATT122*.

pRATT123, containing the *sad-1*$^+$::*gfp*$^+$ fusion, was constructed using the same strategy as for pRATT122 except the template for PCR was cosmid G24:G2, left flank primers were ORP215 and ORP216, right flank primers were ORP217 and ORP218 and *Pst*I substituted for *Bam*HI.

pDLAM259 – *rfp*$^+$::*sms-2*: Was constructed and kindly provided by Dong W. Lee.

Knockout of quelling deficient-2 (qde-2^{RIP40}*).* To obtain an expression vector utilizing the *Sms-2* promoter, inverse PCR with oligonucleotides ORP066 and ORP067 was used to replace the third amino acid codon to the stop codon of the *sms-2*$^{+}$ clone in pDL92a (Lee *et al.* 2003b) with an in-frame *Xba*I site. This ORF deletion was PCR amplified with oligonucleotides ODL159 and ODL160 then subcloned into the *Eco*RI and *Bam*HI sites of the *his-3* insertion vector pJHAM002 (Haag and Aramayo 2003). This plasmid, pRATT36, allows the cloning of ORFs into a unique *Xba*I site to place them under the control of the *Sms-2* promoter and terminator for insertion at the *his-3* locus.

To place the *qde-2* ORF under the *Sms-2* promoter, the third amino acid codon to the stop codon of *qde-2* was PCR amplified with oligonucleotides ORP072 and ORP073 then subcloned in-frame into the *Xba*I site of pRATT36 yielding pRATT39a1.

To obtain strains knocked out for both Neurospora Argonaute genes, pRATT39a1, was integrated at the *his-3* locus in an *Sms-2*^{RIP88} mutant, RPNCR23A. A homokaryon containing the insert, RPNCT28A, was crossed to RANCR06A inducing RIP mutagenesis of the *qde-2* duplications. A progeny containing a RIP mutated version of *qde-2* was isolated. The allele of this strain, *qde-2*^{RIP40}, was PCR amplified from genomic DNA, cloned and sequenced, revealing that the first premature translational stop at amino acid position 115.

Hairpin RNA constructs. To obtain an expression vector utilizing the *ccg-1* promoter, the region from the ATG to 1958 bp upstream were amplified from the cosmid G8:C4 using PCR with primers ORP128 and ORP129. Similarly a 614 bp region from directly downstream the *ccg-1* translational stop was PCR amplified with oligonucleotides ORP130 and ORP131. These fragments were digest with *Xba*I, mixed and ligated. A fusion of the promoter and terminator was

PCR amplified from the ligation with primers ORP128 and ORP131. This fragment was digested with *Bam*HI and *Eco*RI then subcloned into the *Bam*HI and *Eco*RI sites of the *his-3* insertion vector pJHAM002 (Haag and Aramayo 2003). The resulting vector, pRATT66, contains the *ccg-1* promoter and terminator ready to accept an ORF at a unique *Xba*I site. The construct can be used to target integration of the *ccg-1* promoter driving an ORF to a region downstream of the *his-3* locus.

To obtain a inverted repeat of *al-1*, the 1745 bp *Nhe*I/*Bgl*II and 2147 bp *Xba*I/*Bgl*II fragments of cosmid X02:F02 were ligated into the *Bgl*II site of pRATT02 (Pratt and Aramayo 2002). The *Xba*I/*Spe*I fragment of the resulting plasmid was subcloned into the *Xba*I site of pRATT66, thereby placing an inverted repeat of *al-1* under the control of the *ccg-1* promoter and terminator, yielding pRATT67.

pRATT113, used in constructing RPNCT355A, will be described elsewhere.

Bioinformatics

Different multiple sequence alignments were obtained from two global, two local and a combined alignment methods: MAP <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>, ClustalW <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>, PRRN <http://align.genome.jp/prrn/>, Dialign2 <http://bibiserv.techfak.uni-bielefeld.de/dialign/submission.html>, and T-coffee <http://igs-server.cnrs-mrs.fr/~cnotred/Projects_home_page/t_coffee_home_page.html>. We then used T-coffee to essentially create a consensus alignment from these alignments. Sources of the protein sequences used for the alignment of full-length ascomycete SMS-4 orthologs (Figure 18A) and Region A (Figure 18B) are given in Table 5. Alignments were imported into DNA Star (Lazergene) to edit the

alignment and create histograms. Consensus strength of the alignment for the histograms was based on a PAM250 matrix. The large gaps in the Region A alignment were partially compressed by editing in Canvas (Deneba).

For secondary structure prediction and homology search of *S. pombe* SPCC16C4.16c, the full protein sequence was submitted to the BioInfoBank Meta Server <http://bioinfo.pl/meta/queue.pl>. For JScore calculation we included output from BASIC (dist), FFAS03, INUB and ORFeus-2 methods using a single model per server (Bujnicki *et al.* 2001; Ginalski *et al.* 2003; Ginalski *et al.* 2005).

The human ELG protein present in the NCBI protein database lacks Region A, however, the region was detected by doing a tBLASTn search of the human genome using the mouse ELG protein. A gene prediction for ELG containing Region A was present in the NCBI “Map Viewer” of the human genome as Gnomon model: hmm51052. This prediction was used in our alignment. Furthermore, the size of the human ELG isolated as part of the mRNP (Merz *et al.* 2007) was larger than the proteins in the NCBI protein database and thus consistent with the human ELG containing Region A.

Contributions

All work described in the section, “Mutagenesis strategy”, was performed by Kevin Baker and Malcolm McLaughlin. The mutagenesis strategy was designed by Rodolfo Aramayo. Dong Whan Lee, Kevin Baker, Malcolm McLaughlin and Kye-Yong Seong contributed some unpublished materials used in this study (see Table A1). Todd Gruninger constructed TGNC1 strains while on rotation. Robert J. Pratt designed and performed all other experiments described and interpreted the data. The pRATT18 series of plasmids were constructed by RJP

prior to graduate work. RA advised on aspects of some experimental design, data interpretation and editing of the manuscript.

CHAPTER V
CONCLUSION

SUMMARY

In the Research Aims section of Chapter I, three specific objectives were outlined for this research. Each of these objectives became the focus of one of Chapters II through IV. Chapter II presents results showing that the absence of methyltransferase activity from DIM-2 decreases meiotic silencing by homeologous inducers of meiotic silencing, RIP alleles. An attractive possibility is that DNA methylation, either directly or indirectly, interferes with meiotic *trans*-sensing, since meiotic silencing by other inducer classes was unaffected in the mutant. In Chapter III, experiments are described demonstrating that the absence of gene products that are required for the initiation of recombination and the stable chromosome pairing in organisms closely related to Neurospora does not affect meiotic silencing. Thus, the *trans*-sensing that must occur between chromosomal loci occurs through a novel mechanism. In Chapter IV, a novel gene required for meiotic silencing, *Sms-4*, is identified and characterized. SMS-4 is the first component of the pathway that is nuclear localized, and also the first that is not required for ascus development or meiosis. Its nuclear localization is consistent with its playing a role in meiotic *trans*-sensing. These properties imply that meiotic silencing by unpaired DNA is not required for ascus development or meiosis, raising questions about the role of silencing of unpaired DNA in Neurospora biology.

PERSPECTIVES

How is unpaired DNA detected?

First, what is unpaired DNA? Unpaired DNA is DNA that lacks a homologous region at the allelic position of its homologue and that therefore fails to satisfy *trans*-sensing. The details of meiotic *trans*-sensing are unclear and difficult to study given that the only way to detect it thus far is indirectly through meiotic

silencing. By studying the differences between reporter alleles that result in meiotic silencing (i.e., unpaired) and those that do not (i.e., paired), we can gain insight into the rules governing successful *trans*-sensing. Based on these rules, we can speculate on the possible mechanisms of *trans*-sensing.

What are the requirements for pairing?

Interallelic communication must occur between chromosomal loci to satisfy *trans*-sensing (i.e., pair). Conversely, having two copies of a gene at different places in the genome does not satisfy *trans*-sensing, which therefore cannot simply be a gene counting mechanism (Aramayo and Metzenberg 1996). All regions that share homology must satisfy *trans*-sensing to avoid being silenced. Even if two homologous regions are paired, an ectopic homologous region unpaired elsewhere in the genome that is unpaired will lead to the silencing of all three loci (Lee *et al.* 2004; Shiu *et al.* 2001). Note that in this situation, it is presumed that only the novel ectopic copy is unpaired since, in the absence of this copy, *trans*-sensing occurs successfully between the homologous alleles at their canonical location. Alternatives are discussed below. If this ectopic copy is also provided with a homologous region at the same ectopic location, *trans*-sensing is again satisfied (Aramayo and Metzenberg 1996). Even when the only two copies of a reporter are at the same ectopic position *trans*-sensing is satisfied, suggesting that the actual location of the paired regions is unimportant (Aramayo and Metzenberg 1996).

What can be inferred about the homology that satisfies *trans*-sensing? We have some idea of what differences can be detected. Using the reporter *Asm-1*, it was found that indels as small as ~1.4-kbp within the transcribed region can cause low but detectable *cis*- and *trans*-silencing (Lee *et al.* 2004). However, bigger is better since silencing is more efficient if the unpaired DNA is made larger by attachment of non-homologous DNA (Lee *et al.* 2004).

RIP alleles can also trigger silencing in a way that is partially sensitive to DNA methylation. The silencing by indels and ectopic copies, on the other hand, is unaffected by DNA methylation, suggesting DNA methylation affects *trans*-sensing, not meiotic silencing in general. The efficiency of silencing by these RIP alleles in the absence of DNA methylation is proportional to the number of point mutations (Pratt *et al.* 2004). How DNA methylation affects *trans*-sensing of RIP alleles is unclear, but could be due to the point mutations or other effects such as transcriptional repression or chromatin structure (Selker *et al.* 2002). Frameshift mutations and low levels of sequence divergence are do not induce silencing (Aramayo and Metzenberg 1996; Pratt *et al.* 2004; Shiu and Metzenberg 2002). These results imply that small differences may are tolerated during *trans*-sensing or that small differences create poor substrates for silencing.

Certain DNA regions do not trigger detectable silencing even when they lack a homologous region to pair with, including the promoter and untranscribed downstream regions. Silencing is only detected when the unpaired regions contain homology to the transcript of the reporter gene (Lee *et al.* 2004). Three non-mutually exclusive possibilities could explain this observation. 1) Meiotic silencing could only operate on transcripts. For instance, if meiotic silencing operates only on mRNA, then untranscribed regions identified by *trans*-sensing as unpaired would have no phenotypic consequences. 2) Untranscribed regions may be detected as unpaired but be immune to silencing. For instance, meiotic silencing could work by repressing the chromatin in the unpaired region by introducing post-translational modifications in the histone tails. High-resolution ChIP analysis in other organisms has shown that histone modifications within the promoter can differ from those in coding regions (reviewed Li *et al.* 2007). Thus, activated promoter regions may contain histone tail modifications that

prevent their silencing even when they are unpaired or they may recruit activators capable of overwriting the meiotic silencing-introduced histone modification. 3) Untranscribed regions could simply not be compared by *trans*-sensing. This could be the case, for example, if *trans*-sensing occurs through RNA intermediates produced from conventional promoters.

Current evidence is consistent with the first and second alternatives, but it does not exclude the third. There appears to be no direct correlation between conventional transcription and meiotic *trans*-silencing by an ectopic inducer. Specifically, an ectopic inducer lacking its promoter can undergo *trans*-sensing and induce silencing (Lee *et al.* 2004). Furthermore, addition of a small heterologous promoter to an ectopic inducer lacking its native promoter does not increase the observed silencing (Lee *et al.* 2004). These results suggest that conventional transcription is not required for *trans*-sensing, and thus, that untranscribed regions should be susceptible to *trans*-sensing. However, it has not been ruled out that cryptic promoters transcribe these ectopic inducers at low levels to provide the transcription needed for *trans*-sensing. Indeed, inserts of *Asm-1* at the ectopic location used in these experiments contain a mysterious "transcriptionally active element" in the 5'UTR of the canonical transcript, which could be derived from such a cryptic promoter (Lee *et al.* 2004). If so, a promoter fragment containing no transcribed sequence and inserted at the same ectopic location could be transcribed by this cryptic promoter and result in reporter silencing. This has not yet been tested experimentally.

Does meiotic trans*-sensing occur between chromosomes?*

A simple model for *trans*-sensing would be that chromosomal loci are compared through the same DNA-based mechanisms governing meiotic recombination and chromosomal pairing. However, various mutants defective in stable chromosome alignment, double-strand break formation, recombination, and

chromosome synapsis still exhibit locus-specific meiotic silencing (Chapter III). Is it possible, then, that *trans*-sensing occurs through direct comparison of the chromosomal loci? We can hypothesize that intimate, yet unstable, homologue pairing and *trans*-sensing occur early in meiosis and are later stabilized by the initiation of recombination (Figure 15). There are many examples of DSB-independent pairing in meiosis; however, the mechanisms and the intimacy of comparison by these unstable interactions are unclear (reviewed Gerton and Hawley 2005; McKee 2004; Zickler 2006).

An attractive possibility is that homologues pair through unstable DSB-independent, sequence-independent mechanisms providing rough positional information, and that chromosomal loci are then compared in detail through an RNA intermediate. One could imagine that each locus transcribes *trans*-sensing RNAs that remain tethered to the locus. These RNAs could invade the DNA duplex of the homolog, allowing for direct base-pair comparison without the need for double-stranded breaks. Regions that pair could then discard the paired portion of the *trans*-sensing RNAs with no consequence to the cell. Those portions of the *trans*-sensing RNAs that do not pair would then be released as aRNAs to the meiotic silencing machinery, resulting in the silencing of mRNAs sharing sequence identity to the aRNA and the unpaired DNA. Such a model is consistent with the effect of DNA methylation on *trans*-sensing of RIP alleles (Pratt *et al.* 2004), since methylated, transcriptionally repressed regions may be inaccessible to *trans*-sensing RNAs from the homolog. Note that this model does not necessarily require conventional transcription from canonical promoters (Lee *et al.* 2004). This idea is also consistent with the ability of an inversion of a single locus to pair since the rough chromosomal address and sequence are the same (D.W. Lee, unpublished). These proposed aRNA would only correspond to the unpaired DNA, which could explain why unpairing of only the *gfp* portion of a *gfp* fusion allele (i.e., *rsp*$^{+}$*::gfp*$^{+}$/*rsp*$^{+}$*)* results only in the silencing of the *gfp* fusion

transcript (*rsp*$^{+}$*::gfp*$^{+}$) but not the non-fusion transcript (*rsp*$^{+}$) (D.W. Lee, unpublished). Even though the non-fusion transcript shares significant sequence identity to the silenced fusion transcript, the non-fusion transcript does not contain identity to the unpaired DNA or the aRNA that was released to the RNAi machinery.

Could trans-sensing be extra-chromosomal?

Is it possible that *trans*-sensing does not occur through direct chromosomal interaction at all? Perhaps *trans*-sensing occurs in the cytoplasm through RNA molecules produced from the parental genomes. The key complications to this general model are the requirement of chromosomal position and allele-quantity information (Aramayo and Metzenberg 1996). However, we propose two models for how the cell might store this information in extra-chromosomal RNAs that at least make extra-chromosomal *trans*-sensing imaginable.

First, the cell could simply prepare gigantic RNA chromosomes, or at least partial chromosomes. These could be compared at the sequence level by unknown machineries in the cytoplasm. The size of the RNAs would ensure positional information and tight regulation of transcription could ensure quantity information. This could get messy though. There would have to be a mechanism for ensuring that pairing only occurred between these special RNAs, excluding mRNAs. Furthermore, sufficient cytoplasmic volume would have to be reserved for the comparison of a genome equivalent of RNA, presumably without the benefit of compaction afforded to DNA chromosomes.

Secondly, position information could be given by programmed temporal expression of *trans*-sensing RNAs and quantity information controlled with strict transcriptional regulation ensuring that only one message per locus is produced. For instance, a single smaller RNA molecule could be made at programmed loci

from both parents at the same time. Perhaps several loci could be compared at the same time. This smaller pool of RNAs would be compared, and then the next set of RNAs would be produced and compared. RNAs could be made from neighboring loci or different loci on different chromosomes. Quantity information must be present because large segmental duplications of chromosomes result in a meiotic silencing-dependent sterility. These duplications should also result in the duplication of the temporal program and therefore if only sequence was compared, then *trans*-sensing would be satisfied. In other words, the cell would have to detect that there is twice the amount of RNA in the *trans*-sensing pool for the unpaired large segmental duplication to be identified by this mechanism.

A variation on this last model could be a comparison of RNA intermediates with the chromosomal DNA of the mating partner (Figure 25). Again positional and quantity information is required and provided as in the previous model. For instance, one could imagine that the *mat-A* genome produces RNAs from temporally programmed loci, these then go to the *mat-a* genome and look for homology among similarly programmed loci. If homology is found, these loci are then marked as paired and the *trans*-sensing RNAs are destroyed. If homology is not found then the *trans*-sensing RNAs are fed to the meiotic silencing machinery. This would identify extra DNA in the *mat-A* genome as excess RNA and extra DNA in the *mat-a* genome as loci that were not marked with existing RNA. Comparison could be directional as described and directionality could come from the sex or mating-type of the strains. Directionality however requires different substrates be recognized for silencing in the genomes, unpaired RNA from one and unmarked DNA in the other. Alternatively, the parents could take turns in the comparison, which would only require recognition of one substrate. However, in either of these programmed loci models, victory could be to the transposable element that figures out the program and learns how to count, except maybe for the presence of RIP and quelling.

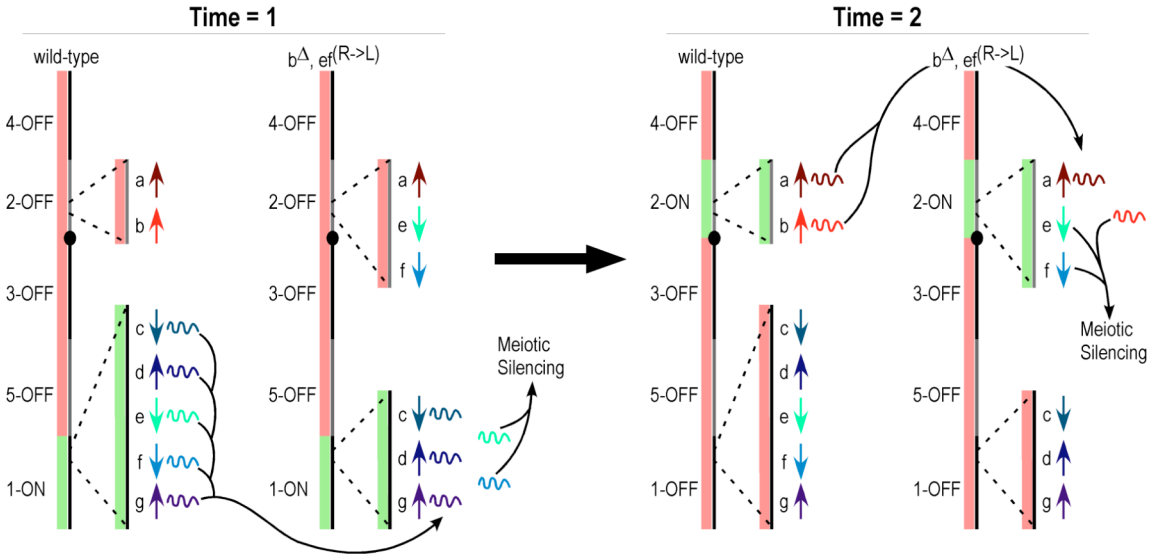

Figure 25. Extra-chromosomal meiotic *trans*-sensing by temporally controlled positional information and strict regulation of transcriptional quantity information. Two homologous chromosomes are depicted as vertical lines with a circle designating the centromere. The chromosomes are divided into 5 temporally programmed regions. To the right of the chromosome is a zoom-in of a few loci (arrows, labeled with letters) within Regions 1 and 2. The parent on the right contains a translocation of genes e and f from Region 1 to replace gene b in Region 2, hence genes b, e and f from the left parent are unpaired and genes e and f from the right parent are unpaired. By the model, at time = 1, Region 1 is ON (green) and all other regions are OFF (red). All of the genes in this region of the left parent produce one RNA molecule. This will then travel to the chromosomes of the right parent and search for regions homologous to this cache of RNAs, but only within ON regions in this parent. As depicted, RNA from genes e and f would fail to find homology and would then trigger silencing of homologous genes. At time = 2, Region 1 is turned OFF and Region 2 is turned ON. During the comparisons with this cache, gene b will lack a homologous region and will be silenced. Additionally, genes e and f will not have an RNA to pair with and these will then be silenced. This is only one possible manifestation of this class of *trans*-sensing mechanisms. See text for alternatives and details.

Overall we still find a chromosome-based *trans*-sensing mechanism simplest and most probable; however, biology is resourceful and could develop solutions to any of the perceived problems with extra-chromosomal *trans*-sensing. Moreover, extra-chromosomal *trans*-sensing has the enticing possibility of pre-karyogamic *trans*-sensing. To maintain the integrity of quantity information within

the multinucleate ascogenous hyphae it seems the nuclei would have to be paired and isolated prior to comparison, for instance in the crozier, which is consistent with the timing of silencing (discussed below) (Aramayo and Metzenberg 1996). The model presented in Figure 25 would additionally requires the controlled and efficient export and import of *trans*-sensing RNAs, making pre-karyogamic *trans*-sensing less likely for this manifestation of the concept. However, extra-chromosomal comparisons that are solely cytoplasmic would only require that export of the RNAs be faithful to the temporal program.

How is unpaired DNA silenced?

Is meiotic silencing a post-transcriptional, RNAi-like mechanism?

Meiotic silencing is most likely an RNAi mechanism given that several of the genes required for the process are homologous to conserved RNAi machinery in Neurospora and other organisms. Specifically, there is an RdRP, *Sad-1*, an Argonaute, *Sms-2* and a Dicer, *Sms-3* (Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu *et al.* 2001). Another component, *Sad-2*, encodes for a perinuclear protein required for efficient localization of *Sad-1* to the nuclear periphery during meiotic prophase I (Shiu *et al.* 2006). We can propose a tentative model for meiotic silencing based on the limited information we have from Neurospora and the known roles of orthologs of these gene products in other systems. We hypothesize that *trans*-sensing identifies unpaired DNA and generates an aberrant RNA (aRNA) molecule from the unpaired region. The nature of the aberrancy is unknown, but this molecule is somehow differentiated from the RNA from paired regions since only it is a substrate for the initiation of meiotic silencing. This aRNA is then transported to the nuclear periphery where the RdRP-*Sad-1* converts the single stranded aRNA to dsRNA. This dsRNA would then be diced into siRNAs by the Dicer-*Sms-3*. A complex containing Argonaute-*Sms-2* would then use the siRNAs to target cleavage then degradation of

mRNAs with sequence complementarities. This is a simple model but how much of it corresponds to reality?

A post-transcriptional mechanism is supported by five pieces of evidence. 1) RNAi-related gene products are required (Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu *et al.* 2001). 2) Steady state levels of mRNA from unpaired DNA are reduced during meiosis (Lee *et al.* 2004). 3) Silencing can act in *trans* on paired DNA (Lee *et al.* 2004; Shiu *et al.* 2001). 4) Silencing persists in the absence of unpaired DNA (Figure 16, Freitag et al. 2004). 5) Silencing is only detected when the unpaired regions are homologous to mRNA (Lee *et al.* 2004). We note that while all of these are consistent with a post-transcriptional mechanism, all but the requirement of RNAi-related genes are also consistent with other models discussed below.

There is no direct evidence that unpaired DNA is silenced post-transcriptionally directly through an RNAi-like mechanism. Only *Sad-1* was isolated by an unbiased forward genetics approach. *Sms-2* and *Sms-3* were isolated by reverse genetics, which is intrinsically biased. Two additional complementation groups have been characterized by forward genetics, *Sad-2* and *Sms-4*. Neither of these have known roles in RNA silencing in other organisms nor do they have predicted functions that would necessarily implicate them in RNA silencing. SAD-2 has no motifs to hint at a function, and SMS-4 looks most like an mRNA splicing factor (Chapter IV). Therefore, the genetic basis of our RNAi-based model is really only the unbiased isolation of one gene.

Despite this limited genetic information, *Sad-1* does not provide the only evidence for an RNAi-based mechanism. For instance, the mutant phenotype seen in crosses exhibiting meiotic silencing is most likely related to the observed loss of transcript corresponding to the reporter gene. When one or two copies of

Asm-1 are unpaired in *cis,* all transcripts corresponding to a shorter, ascus-specific transcript are lost (Lee *et al.* 2004). Additionally, an RNAi-based mechanism offers a simple explanation for how unpaired DNA in one part of the genome silences paired DNA: since RNAi only has sequence information, it naturally does not know if the target mRNA was from a paired or unpaired region. Furthermore, an RNAi-based mechanism allows for the observed persistence of silencing after the homologues have separated at Meiosis I and there is presumably no more meiotic *trans*-sensing (Freitag *et al.* 2004a). Finally an RNAi mechanism explains why only unpaired regions sharing sequence identity to a reporter transcript result in silencing of the reporter (Lee *et al.* 2004), since only the transcript is the target for RNAi.

It is difficult to detect the siRNAs that would provide direct evidence of an RNAi mechanism because of the complexity of the fruiting bodies in which the silencing occurs. Additionally, all characterized mutants other than *Sms-4* have meiotic or ascus-development defects that prevent the production of progeny when both parents are mutant (Chapter IV Alexander *et al.* 2007; Lee *et al.* 2003b; Shiu *et al.* 2001). Thus, it remains an open question whether these gene products are directly involved in meiotic silencing. For instance, RNAi might regulate a class of genes that are true molecular components required for meiotic silencing by unpaired DNA. In the absence of *Sad-1*, these components would not be expressed or only at low levels. These, combined with the paucity of RNAi components isolated by unbiased forward genetics, provide reasons to doubt this post-transcriptional RNAi-based mechanism. Below, we will explore alternatives and extensions of this model.

Is meiotic silencing a transcriptional silencing mechanism?

The decreased steady state level of mRNA from an unpaired reporter is consistent with the idea that silencing occurs at the transcriptional or post-

transcriptional levels. In the first instance, unpaired DNA could be directly coated with repressive chromatin. In the second scenario, RNAi products could go to the nucleus and destroy nascent target mRNAs.

The first model has two difficulties. The first is that not only the unpaired DNA itself is silenced, but homologous paired regions are silenced in *trans*. Another is the requirement for transcript homology in the unpaired region for efficient silencing. Apparently a gene cannot be silenced by unpairing its promoter (Lee *et al.* 2003b). If unpaired promoters are targeted by repressive chromatin, it is not clear why gene expression would not be affected. However, it could be that only transcribed regions are effectively targeted as discussed above.

The second model is perhaps easier to envision, since this type of RNAi-mediated transcriptional repression occurs in *S. pombe* and plants (reviewed Bernstein and Allis 2005; Lippman and Martienssen 2004). In *S. pombe*, the RNAi machinery is required to establish heterochromatin at centromeres as well as for post-transcriptional silencing (Hall *et al.* 2002; Sigova *et al.* 2004; Volpe *et al.* 2002). Even in *S. pombe*, the dependence on RNAi for transcriptional silencing is locus-dependent (Hall *et al.* 2002; Jia *et al.* 2004). During vegetative growth in Neurospora, however, transcriptional and post-transcriptional silencing of repeated RIPped transposable elements occur independently (Chicas *et al.* 2004). Furthermore, there are no obvious vegetative growth defects in any of the Neurospora RNAi mutants, and establishment of heterochromatin on RIPped DNA is RNAi-independent (Freitag *et al.* 2004b). However, the core RNAi components required for meiotic silencing are phylogenetically closer to their orthologs in *S. pombe* than to their vegetative paralogs in quelling (Galagan *et al.* 2003). Additionally, SMS-5, a newly identified component of meiotic silencing, has a SET-domain (D.W. Lee, unpublished). Proteins containing SET-domain

modulate chromatin structure by the post-translational methylation of lysines in histone tails (reviewed Dillon *et al.* 2005; Kouzarides 2007).

But is post-transcriptional silencing needed? From the time the haploid nuclei are isolated in the penultimate cell of the crozier until the progeny nuclei are encapsulated in to the ascospores, all nuclei share the same cytoplasm (Raju 1980). If the RNAi components are cytoplasmic, which *Sad-1* and *Sms-3* are (Alexander *et al.* 2007; Shiu *et al.* 2006), and the RNAi machinery is capable of maintaining the silencing, then there is no obvious need to transcriptionally silence the unpaired region. Perhaps transcriptional silencing provides an extra layer of protection or meiotic RNA silencing alone is unable to maintain silencing in the absence of continued unpaired DNA after the first meiotic division.

If silencing is transcriptional, then RNA-FISH experiments, in combination with immunolocalization of RNA Pol II, could be used to determine whether large regions of unpaired DNA are transcribed. This method could also be used to determine if there is transcriptional silencing in *trans*. A pilot experiment would be to unpair several introns of a reporter gene. Introns are only present in the nucleus, and the silencing of the reporter would suggest that at least some silencing occurs in the nucleus. Introns are not an effective substrate for quelling, which is argued to be cytoplasmic (Cogoni *et al.* 1996).

Is meiotic silencing of unpaired DNA an active process?

Both of the above describe active mechanisms to silence wicked unpaired DNA, but could we have this backwards? Is it possible that all DNA is off but only paired DNA is turned back on, akin to transvection in Drosophila (Lewis 1954; Wu and Morris 1999)? This is difficult to reconcile with *trans*-silencing, since this would require that paired regions also be excluded from expression if a homologous region is unpaired elsewhere. *Trans*-silencing always involves a

gene duplication in one of the parents; perhaps the extra DNA interferes with the pairing of the other copies? However, the presence of a gene duplication per se cannot be responsible for silencing in meiosis since, when both parents contain the same paired duplication there is no silencing. The remaining possibility is that a copy that fails to activate can interfere with the activation of paired regions (discussed below), but this would arguably be an active process. Therefore, it seems that unpaired DNA must be actively silenced not simply excluded from activation. We note that the apparent necessity of an active *trans*-silencing does not preclude the existence of passive silencing in *cis*.

How is paired DNA silenced by unpaired DNA?

During *trans*-silencing, one parent contains an extra ectopic copy of a gene that is absent from this new position in the other parent. As a result, the two copies at their canonical location can pair during meiosis, however the ectopic copy fails to pair resulting in meiotic silencing of all copies. The simplest model for this silencing is that the unpaired DNA produces a diffusible *trans*-acting signal from unpaired DNA, which then targets silencing of all homologous regions (Figure 26A). RNAi offers a simple mechanism for this silencing, since it would be blind to the pairing status of the locus that created the mRNA. Under this model, paired regions are innocent bystanders of the silencing of the ectopic copy. Contrary to the RNAi model, silencing mechanisms (above) that lack a diffusible signal make silencing paired copies difficult, yet not unimaginable.

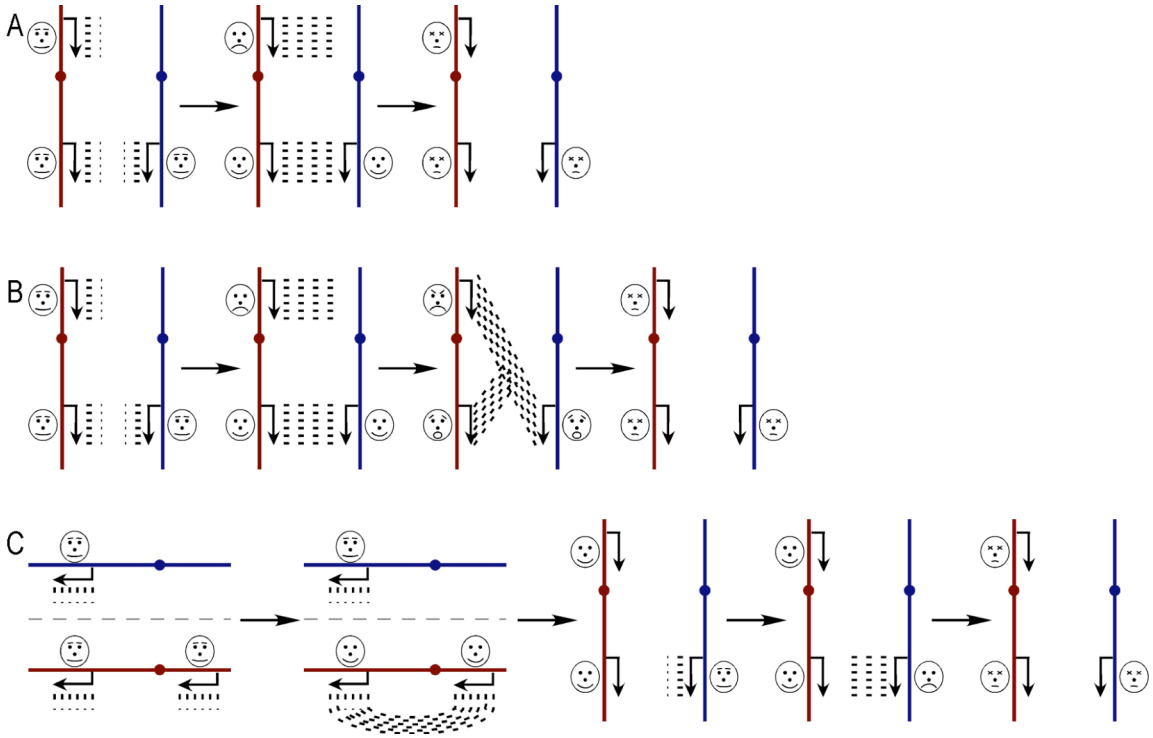

Figure 26. Models for the silencing of paired DNA. A) Ectopic unpaired DNA triggers silencing of all regions of sequence identity. Two homologous chromosomes (red and blue) are compared by meiotic *trans*-sensing (dashed lines). The two copies at allelic positions satisfy *trans*-sensing (smiley faces), but the ectopic copy does not satisfy *trans*-sensing (sad face) and triggers meiotic silencing of all copies (dead faces) without any direct interaction with the paired copies. B) Ectopic unpaired DNA directly silences or interferes with the pairing of other copies. As above, *trans*-sensing is strictly limited to interaction between allelic positions on homologous chromosomes and again *trans*-sensing is satisfied at the allelic copies and fails at the ectopic copy. The ectopic copy is silenced yet remains capable of *trans*-sensing (angry face). The restriction of *trans*-sensing between homologs is then removed, allowing *trans*-sensing between the ectopic copy and any other copy in the genome (alarmed faces), similar to *trans*-sensing during RIP. Pairing of the ectopic copy with one of the other copies could cause unpairing of the normally paired loci and/or could directly transfer a silent epigenetic state. C) Pre-meiotic and meiotic counting. Prior to karyogamy (vertical dashed lines) RIP-associated *trans*-sensing occurs. Copies that were paired are no longer substrates for *trans*-sensing. Following karyogamy *trans*-sensing occurs only between allelic positions. The copy that lacked a pre-karyogamic pairing partner fails to satisfy *trans*-sensing, resulting in the silencing of all homologous copies as in A or B.

Are unpaired ectopic copies directly involved in the silencing of paired regions?

Is it possible that the ectopic copy plays a more direct role in the silencing of the paired copies? One could postulate that unpaired regions themselves are activated to identify homologous regions perhaps through the same *trans*-

sensing mechanism that identified it as unpaired, for instance, a two stage *trans*-sensing mechanism (Figure 26B). First unpaired DNA is identified by the lack of homology on the opposing chromosome and sentenced to silencing. Then these unpaired regions are activated to *trans*-sense homologous regions elsewhere in the genome. Two possible consequences of this *trans*-sensing arise. First, this ectopic pairing could interfere with the *trans*-sensing between the other loci, resulting in all loci being unpaired and silenced. Secondly, pairing with this ectopic copy could directly silence the other copies, perhaps by transfer of the silenced state. The latter requires a shift from silencing *trans*-sensing negative loci during the first step then silencing *trans*-sensing positive loci in the second step. While, seemingly overly complicated, it is not too far of a stretch since this second *trans*-sensing and silencing program would be analogous to that used by the pre-meiotic RIP program, except without the effecter point-mutation step.

This model could predict a delay in the timing of *cis* vs. *trans*-silencing. It's unclear if such a delay, even if existent, would be long enough to detect. Additionally, if *trans*-sensing and meiotic silencing are strictly temporally isolated any difference would be undetectable using meiotic silencing as the output. In a strictly post-transcriptional RNAi model however there should not be a difference in timing, assuming no difference in the timing of *trans*-sensing at the loci.

Can pre-meiotic trans-sensing substitute for meiotic trans-sensing?
Indeed, it is formally possible that there is communication between RIP and meiotic *trans*-sensing (Figure 26C). Imagine that, following fertilization, each locus is tagged with a *trans*-sensing flag. This flag is a necessary substrate for *trans*-sensing. Pre-meiotically, flags that pair by *trans*-sensing are targeted for RIP and the flag removed. In meiosis, *trans*-sensing is restricted to comparing flagged loci on homologous chromosomes. If both loci have flags, then they are paired. If either lacks a flag, then only the one with the flag undergoes *trans*-

sensing but will be unpaired because its partner lacks a flag. Meiotic silencing would then be trigged, silencing all copies possibly through the mechanisms illustrated in Figure 26A or Figure 26B. However if both loci lack flags because both were identified previously pre-meiotically, they do not undergo *trans*-sensing at all and therefore are unable to trigger meiotic silencing.

A simple test for this model would be to cross two parents containing ectopic insertions at different non-allelic loci. Unlike other models presented, this model predicts no silencing in these crosses. Note that *trans*-silencing occurs efficiently in *rid-1* mutants (Chapter IV), so the mutations, DNA methylation and repressive chromatin associated with RIP cannot be required for flag removal, rather only the satisfaction of *trans*-sensing is postulated. Additionally, RIP varies in efficiency but typically only occurs in ~50% of duplication containing nuclei. However this is scored by loss of function of a duplicated gene and thus is not necessarily a measure of *trans*-sensing efficiency.

This is a modified counting mechanism that differs from a simple counting mechanism in that it incorporates homologous chromosome comparison. In addition, a counting mechanism is proposed to exist for RIP based on the high frequency at which triplicate containing strains only RIP a single duplication (Fincham *et al.* 1989). The critical problem with this and other counting mechanisms for meiotic silencing is that it would be ineffective in detecting and silencing extra DNA so long as both parents have an even copy number of the same extra DNA, however this makes assumptions about the critical role of meiotic silencing.

There is at least one inconsistency with this last model and the existing data. By this model, it is unclear why silencing is better when an ectopic insertion is made larger by fusion to a large region of heterologous sequence (Lee *et al.* 2004). In

the direct and indirect silencing models (Figure 26A&B), one might imagine that the larger loop of unpaired DNA is simply detected with greater efficiency in general or during the first step of the direct model. However the counting model (Figure 26C) is more difficult to reconcile with this evidence because the allele that is unpaired does not have the heterologous sequence; therefore, it is hard to imagine how the heterologous insertion would affect pre-meiotic *trans*-sensing. The heterologous region would be unpaired during meiosis but this should not affect dominance since silencing does not seem to spread to neighboring regions (Kutil *et al.* 2003). We can only conclude that this model and/or our imaginations are flawed.

When and where is unpaired DNA detected and silenced?

Probably, *trans*-sensing and meiotic silencing occur after nuclear fusion and during meiosis, since this is the first time that homologous chromosomes could directly meet (Aramayo and Metzenberg 1996). However as discussed above, pre-karyogamic *trans*-sensing mechanisms are possible. Since there is no direct measure of *trans*-sensing, pinning down its timing is not currently possible, however, since unpaired DNA needs to be identified before it can be silenced, *trans*-sensing must occur at least before meiotic silencing. Currently, our best approximation of timing comes from looking at the signal from of a *hH1*$^{+}$::*gfp*$^{+}$ fusion during the course of sex. These experiments are intrinsically flawed in that silencing is only seen when protein is lost and it is unknown how long the hH1::gfp protein lasts during meiosis.

With this caveat in mind, meiotic silencing does not seem to occur efficiently immediately following fertilization since GFP signal is visible in fertilized tissues three days after fertilization even when *hH1*$^{+}$::*gfp*$^{+}$ is unpaired in *cis* (Figure 16). However around the time of the formation of the first meiotic asci, GFP signal is lost only when *hH1*$^{+}$::*gfp*$^{+}$ is unpaired, suggesting meiotic *trans*-

sensing and meiotic silencing occur very early in ascus development (Figure 16). Consistent with this, in crosses where actin is unpaired in *trans*, pre-zygotic dikaryotic tissues still have actin (Shiu *et al.* 2001). There however is no conclusive evidence that meiotic silencing does not occur in the crozier prior to karyogamy.

There is evidence however suggesting that meiotic silencing is ascus autonomous. First, unpairing of actin and beta-tubulin in *trans* results in meiotic silencing, but apparently only results in meiotic defects (Shiu *et al.* 2001). Presumably if meiotic silencing were capable of spreading from the meiotic ascus, to all of the perithecial tissues, the loss of these products would prevent the formation of future asci. Additionally, all reporter genes are not silenced to 100% efficiency, resulting in a mixture of asci showing silencing and some not, rather than the expected homogenous phenotype if all of the perithecial tissue shared the same silencing (Figure 11). Further evidence comes from an experiment where the perithecia contained a mixture of three nuclei: two different maternal nuclei and a paternal nucleus (Shiu and Metzenberg 2002). One maternal nucleus contained a deletion of a reporter and the other did not. Progeny arising from the nucleus lacking the deletion (wild-type) could be followed by the presence of a metabolic marker. When meiotic silencing was observed in these mixed perithecia, it only occurred in asci containing the reporter deletion. Therefore, meiotic silencing was unable to silence a paired reporter in neighboring asci. Together, these data suggest that meiotic silencing is ascus autonomous, but do not necessary imply that meiotic silencing is initiated after karyogamy, only that it occurs in a limited space unable to spread to neighboring tissues. Even if prevented from spreading to neighboring tissues, if meiotic silencing occurs before karyogamy, would not the silencing of actin and beta-tubulin block karyogamy? Not if sufficient protein was there prior to silencing of the mRNA.

An odd discrepancy does seem to exist however. When *hH1*$^+$::*gfp*$^+$ was unpaired in *cis* from its native promoter, all GFP including that in the young pre-zygotic tissue was lost (Figure 16). This cannot be due to the changes in *hH1*$^+$::*gfp*$^+$ expression because signal is detected in these tissues when unpaired in a meiotic silencing mutant or paired. However, unpairing actin and other genes in *trans* does not seem to result in silencing of this younger tissue. Could this be evidence of a delay between *cis*- and *trans*-silencing? This is currently unclear as the analysis of these crosses was not rigorous enough and/or not reported thoroughly enough to conclude anything at this point. It is clear however from the evidence cited above, that even if *cis*-silencing can spread to pre-karyogamic tissues, the decision to silence in the ascus is ascus autonomous. In addition, there are other explanations for the potential discrepancy. For instance, perhaps the hH1 protein in the young tissue is usually supplied by the asci at the later stages of development. It would be of particular interest to see if DNA polymerase or other pre-meiotic gene products can be silenced in young tissue in *cis.*

After it is initiated early in ascus development, meiotic silencing persists through out the course of meiosis and through the post-meiotic mitosis. Then it appears to stop some time after spore formation. When exactly is not clear. The resetting has only been directly seen using a fusion of *hH1*$^+$ to *gfp*$^+$ and so the time at which the fusion is seen again is a sum of the time required for meiotic silencing to turn off, *hH1*$^+$::*gfp*$^+$ to be transcribed, translate, and fold properly. In addition, since there is no evidence that *hH1*$^+$::*gfp*$^+$ is transcribed at all during meiosis, it is theoretically possible that meiotic silencing is turned off well before ascospore formation but that *hH1*$^+$ simply is not turned on until then. The pattern of reactivation is the same for *hH1*$^+$ expressed under its promoter (Figure 16) and under the heterologous *ccg-1* promoter (Freitag *et al.* 2004a), however both

reporters share this same flaw. Importantly however, meiotic silencing is turned off. And perhaps the more interesting question is why? Why would Neurospora not want to continue silencing potential transposable elements in the next generation?

What is the role of meiotic silencing?

We do not know what the role of meiotic silencing is. Based on the phenotypes of meiotic silencing mutants and the role of RNAi in other organisms, we will speculate on three possible roles for meiotic silencing in Neurospora biology, *viz*., genome defense, gene regulation and speciation.

Silencing of transposable elements

As we have modeled above (Introduction, Figure 2), meiotic silencing could be important in ensuring that transposable elements do not spread during meiosis. For a transposable element, sex can represent a great opportunity to move into novel genetic backgrounds with minimal risk to the host. In Neurospora, sex is not unique in this opportunity however, since mycelia are syncytial and undergo frequent hyphal fusions between strains of the same mating-type and heterokaryon incompatibility groups. These mating-type and heterokaryon incompatible loci however represent a potential barrier to the spread of transposable elements to nuclei that differ at these loci. Fusion between such heterokaryon incompatible strains results in severely reduced growth rate or death (Glass and Dementhon 2006; Glass *et al.* 2000). These mechanisms are however turned off during sex and meiosis, creating the opportunity for transposable elements to move into novel backgrounds forbidden during vegetative growth. There is no direct evidence for the effectiveness of meiotic silencing or heterokaryon incompatibility in controlling the spread of transposable elements; however, given the known properties, they arguably should at least to some extent.

Regulation of sexual-development genes

Another possible role for meiotic silencing is in gene regulation. All meiotic silencing components implicated in RNAi are required for sex. *Sms-2* and *Sms-3* mutants have an early block in sex; fertilization occurs however, development is blocked before or immediately following karyogamy. Meiotic asci are never produced in these mutants (Chapter IV; Alexander *et al.* 2007; Lee *et al.* 2003b). *Sad-1* is blocked later at around pachytene or diplotene of meiosis I (Shiu *et al.* 2001). While the block in *Sms-2* and *Sms-3* is earlier, we cannot rule out a role for these additionally at the same time point as *Sad-1*. Indeed *Sms-3* and *Sms-2* are perinuclear with along with *Sad-1* (Alexander *et al.* 2007) and *Sad-1* is required for normal *Sms-3* perinuclear localization (Dong Whan Lee, unpublished observation).

What are they doing that is so important? A simple hypothesis is that they are required for the silencing of particular genes during development. Could this be unpaired DNA? The *mating-type* idiomorphs are the only known loci that are always unpaired in meiosis (Glass *et al.* 1990; Glass *et al.* 1988). It has been suggested that these genes are protected against meiotic silencing because their expression is required for meiosis (Shiu *et al.* 2001). The only evidence lies in a single experiment. A strain containing an engineered translocation of *mating-type A* to a different chromosome is functionally *mat-A* in fertility; however, crosses between this strain and a *mat-a* strain are sterile and this sterility is at least partially *Sad-1* and *Sms-4* dependent (Figure 21B (Shiu *et al.* 2001). The simplest explanation is perhaps that the unpairing of *mat-A* causes sterility because it is required but silenced by meiotic silencing. This implies an immunity of the *mating-type* loci to silencing when at their own chromosomal position since they are normally unpaired but not silenced. By this simple scenario, the role of meiotic silencing cannot be to silence unpaired *mating-type*.

However, is it this simple? One could imagine that the immunity of the *mating-type* loci is not to meiotic silencing *per se*, but to chromatin-mediated persistence of transcriptional meiotic silencing as described above. Perhaps *mating-type* is normally, and necessarily, silenced early in meiosis by RNAi. However, the chromatin context of the *mating-type* loci may prevent their imprinting and transcriptional silencing later in meiosis. Thus, when *mating-type* is unpaired ectopically it gets silenced like it should initially, but fails to reactivate due to the persistence of meiotic silencing at the ectopic location. In crosses heterozygous for a dominant *Sad-1* mutation, perhaps asci that reach the appropriate balance of silencing *mating-type* and *Sad-1* itself produce progeny, while many still fail due to a persistence of silencing of *mat-A*. In crosses homozygous for *Sad-1*, *mating-type* would then be miss-regulated, preventing proper ascus development.

Alternatively, one could hypothesize that establishment of immunity itself is *Sad-1*-dependent. Perhaps, meiotic silencing induced by the unpaired idiomorphs, along with *cis*-acting factors specifically at the *mating-type* loci, establish a chromatin structure that masks the unpaired *mating-type* idiomorphs. This could be analogous to RNAi-mediated heterochromatin formation at the silent *mating-type* loci in *S. pombe*, where establishment is dependent on RNAi and on other factors at *cis*-acting sequences (Hall *et al.* 2002; Jia *et al.* 2004). By this model, loss of some components, e.g., Sad-1, would result in failure to establish immunity and meiotic defects.

A more detailed analysis of the regulation of *mating-type* genes during meiosis and in meiotic silencing mutants is desperately needed. It is of particular interest since this type of RNAi-mediated transcriptional imprint of sex-determining

regions could be the primordial mechanism of sex chromosome inactivation in mammalian spermatogenesis (Handel 2004; Turner 2007).

Is there any other unpaired DNA? It is possible, since DNA sequence may not be the only trigger for meiotic silencing. DNA methylation either directly or indirectly appears to affect *trans*-sensing between methylated and unmethylated alleles (Pratt *et al.* 2004). Such epigenetic modifications could be differentially programmed at loci of the parental genomes perhaps in a mating-type dependent manner. Differential DNA methylation is unlikely to be the culprit since meiosis is normal in mutants of the DNA methyltransferase, *dim-2* (Kouzminova and Selker 2001), however other modifications cannot be excluded. Indeed mutants in the histone methyltransferase, *dim-5*, like *Sad-2* mutants, produce few successful meioses (Tamaru and Selker 2001).

One meiotic silencing mutant perhaps sheds doubt on these models. *Sms-4* is required for meiotic silencing but is not required for meiosis. It encodes a nuclear protein that probably binds RNA. Epigenetically-triggered silencing by Rsp^{RIP93} is *Sms-4*-dependent, demonstrating that at least some epigenetic triggers still require *Sms-4*. However, induction by other imprints could bypass the *Sms-4* requirement. Full silencing of the ectopic *mat-A* gene is dependent on *Sms-4*, suggesting that the gene-product itself is not immune to *Sms-4*-dependent silencing. For *mating-type* to be necessarily silenced as unpaired for development, something must make it special to be *Sms-4*-independent, for instance the immunity. Perhaps initial establishment of silencing in general is *Sms-4*-independent, i.e., perhaps *Sms-4* has a role specifically in maintenance of silencing. Time course experiments suggest that silencing of unpaired *hH1*$^{+}$*::gfp*$^{+}$ at all stages of ascus development requires *Sms-4*. However, we could have missed an early *Sms-4*-independent silencing due to persistent

HH1::GFP protein produced prior to the induction of meiotic silencing. Additionally, we cannot exclude the possibility that *Sms-4* only acts upon a subset of genes to which these silenced hypothetical development genes do not belong (Chapter IV).

Alternatively, perhaps meiotic silencing is required to silence developmental gene that are not unpaired at all. It is conceivable that microRNAs originating from paired regions regulate critical events during sex through the RNAi apparatus. *Sms-4* may be critical only for the meiotic silencing by unpaired DNA but not for other substrates of RNAi. The localization of *Sms-4* to the nucleus is consistent with a role in *trans*-sensing or as a conduit between meiotic silencing and meiotic *trans*-sensing, rather than a general component of RNAi which appears to be perinuclear and cytoplasmic (Chapter IV). We have unfortunately been unable to detect promoter-dependent, hairpin-directed RNAi in meiosis, which could have offered insight.

Aid in speciation

Based on the increased fertility of interspecific crosses containing a dominant *Sad-1* mutation, meiotic silencing appears to be a barrier to interspecific crosses, at least among some Neurospora species (Shiu *et al.* 2001). Thus meiotic silencing may facilitate reproductive isolation and speciation. For instance, imagine a rearrangement resulting the replacement of the 5’ or 3’ untranslated regions of a gene required for meiosis. Strains containing this allele would have reduced fertility when crossed to the wild-type population, however, so long as the rearrangement did not significantly disrupt activity of the gene, its progeny would be fertile among themselves. The dominance of the rearrangement would ultimately determine the degree of reproductive isolation. If isolated long enough, other changes could occur that strengthen the isolation.

Importantly, spontaneous deletions in the meiotic silencing machinery could allow for occasional gene flow between these new species. In a sense, this gives Neurospora the best of both worlds. Rapid speciation could be good as it minimizes genetic exchange with those less worthy while still allowing some progeny to exchange genetic materials with related species.

Note that this almost certainly has to be a replacement of the 5' or 3' regions of the gene since both transcripts need to lack homology on the opposite chromosome for both copies to be silenced. Insertions into the transcript are typically recessive probably because only the copy containing the insert lacks homology. For instance, the GFP fusion constructs used in our lab insert ~3 kb of DNA onto the end of the coding region. In crosses to a wild-type strain these fusion alleles are themselves unpaired and readily silenced however do not extensively silence their wild-type pairing partner and are thus recessive (D.W. Lee, unpublished). By the same notion, a simple deletion in one strain that left the gene functional could still be recessive since the deletion allele would still have full homology in the wild-type allele.

How might such a rearrangement occur? If a transposable element downstream of a gene were to jump into the 3' untranslated region of this gene thereby creating a direct repeat of the transposable element, then recombination between these repeats and eviction of the intervening 3' UTR would result in just this type of replacement. If the intervening region is large, containing non-essential genes, then the resulting allele could be very dominant. Alternatively, tandem duplication of the 3' UTR, perhaps with the addition of downstream regions, followed by RIP could change the sequence of the 3'UTR sufficient to effect *trans*-sensing of alleles. However this requires not only that the allele with the RIPped 3'UTR be functional but also that the RIPped 3'UTR be transcribed.

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APPENDIX A

Table A1: Fungal strains used in this study

Name[a]	Genotype[b]	Origin
ACNC1	*Rsp*; inl89601 A	RLM81-31 X RLM84-37
ACNC9	*his-3*1-234-723, *Rsp, un-18; inl*89601 *A*	DJNC2 X RANCR5A
DE41 a	*pe, fl; rca-1*Δ*::hph*+ *a*	(Shen *et al.* 1998), D.J. Ebbole Lab
DJNC2	*al-2, Rsp, un-18 a*	David J. Jacobson
DLNCT62A&C	*his-3::hph*+*::tk*+*::lpl*Δ(5192-6046)*; inl*89601 *A*	(Lee *et al.* 2003a)
DLNCR83B	*his-3*1-234-723::*lpl*Δ(5192-6046)::*hph*+::*tk*+; *inl*89601 *a*	(Lee *et al.* 2003a)
DLNCR88A	*his-3*1-234-723*; inl*89601*; Sms-2*RIP88 *a*	(Lee *et al.* 2003b)
DLNCR93A	*his-3*1-234-723, *Rsp*RIP93*; inl*89601*A*	To be described elsewhere
DLNCR94A	*his-3*1-234-723, *rsp*RIP94*; inl*89601 *A*	To be described elsewhere
DLNCR99A	*his-3::lpl*Δ(5192-6046)*::hph*+::*tk*+, *Rsp*; *inl*89601 *a*	To be described elsewhere
DLNCR100A	*his-3*1-234-723*; inl*89601*; Sms-2*RIP88 *A*	(Lee *et al.* 2003b)
DLNCR123A	*Sad-1*RIP64, *his-3*1-234-723*; inl*89601*; Sms-2*RIP88 *A*	DLNCR88 X DLNCR69
DLNCR142A	*his-3*1-234-723*; fl*p*; inl*89601*; Sms-2*RIP88 *A*	DLNCR100A X RANCR50A
DLNCR147A	*fl*p*; inl*89601*; Sms-2*RIP88 *a*	Sibling DLNCR142A
DLNCR212A	*his-3*1-234-723, *Rsp*Δ(2516-6316)::*hph*+; *inl*89601 *a*	To be described elsewhere
DLNCR213A	*his-3*1-234-723, *Rsp*Δ(2516-6316)*::hph*+*; inl*89601 *a*	DLNCT212 X RANCR6A
DLNCR215A	*his-3*1-234-723, *Rsp*Δ(2516-6316)*::hph*+*; dim-2*1 *a*	DLNCT214 X RPNCR75A
DLNCR245A	*rid-1*RIP245, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6046)*; inl*89601 *A*	To be described elsewhere
DLNCR246A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6046); *inl*89601 a	To be described elsewhere
DLNCR315A	*his-3*1-234-723; *mus-51*RIP315; *inl*89601 *A*	To be described elsewhere

Table A1. Continued

Name[a]	Genotype[b]	Origin
DLNCR316A	*his-3*1-234-723; *mus-51*RIP316; *inl*89601 *a*	To be described elsewhere
DLNCR380A	*rid-1*RIP246, *his-3::hph*$^{+}$*::tk*$^{+}$*::lpl*$^{\Delta}$(5192-6046); *inl*89601; *Sms-2*RIP88 *a*	DLNCR100A X DLNCR246A
DLNCT115A	*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Rsp*[1123-6375], *Rsp; inl*89601 *A*	To be described elsewhere
DLNCT117A	*his-3*1-234-723; *inl*89601; *qde-1*$^{\Delta}$*::hph*$^{+}$ *A*	To be described elsewhere
DLNCT212A	*his-3*1-234-723, *Rsp*$^{\Delta}$(2516-6316)*::hph*$^{+}$; *inl*89601 *A*	Transformation of RANCR5A with pDL96
DLNCT214A	*his-3*1-234-723, *Rsp*$^{\Delta}$(2516-6316)*::hph*$^{+}$; *dim-2*^{1} *A*	Transformation of RPNCR77A with pDL96
DLNCT290A	*rid-1*RIP245, *his-3*$^{+}$*::Asm-1*$^{+}$[9336-3430]; *inl*89601 *A*	Transformation of DLNCR245A with pKYAM011 (Lee *et al.* 2004)
DLNCT291A	*rid-1*RIP246, *his-3*$^{+}$*::Asm-1*$^{+}$[9336-3430]; *inl*89601 *a*	Transformation of DLNCR246A with pKYAM011 (Lee *et al.* 2004)
DLNCT389A	*rid-1*RIP246, *his-3*$^{+}$*::ccg-1(p)::rfp*$^{+}$*::sms-2; inl*89601; *Sms-2*RIP88 *a*	Transformation of DLNCR380A with pDLAM259
DLNCT391A	*rid-1*RIP246, *his-3*$^{+}$*::ccg-1(p)::rfp::sms-3*$^{+}$; *Sms-3*RIP65; *inl*89601 *a*	Transformation of DLNCR381A with pDLAM262
FGSC 2420	*a*	FGSC[d]
FGSC 2920	*mei-1 a*	(Smith 1975); FGSC[d]
FGSC 4564	*ad-3B*12-17-114, *cyh-1*R(KH52r) *a*m1	FGSC[d]
FGSC 5146	*mus-9*FK104 *A*	FGSC[d]
FGSC 5888	*Mei-2*ALS181; *nic-3*Y31881 *a*	(Schroeder and Olson 1983); FGSC[d]
FGSC 6187	*mei-3*C325* *A*	FGSC[d]
FGSC 6188	*mei-3*C325* *a*	FGSC[d]
FGSC 6409	*mus-11*FK117 *A*	FGSC[d]
FGSC 6418	*mus-21*FK120 *A*	FGSC[d]
FGSC 8740	*Sad-1*$^{\Delta}$::*hph*$^{+}$ *A*	(Shiu *et al.* 2001); FGSC[d]

Table A1. Continued

Name[a]	Genotype[b]	Origin
FGSC 8741	*Sad-1*Δ*::hph*+ *a*	(Shiu *et al.* 2001); FGSC[d]
FGSC8592	*dim-2*1 *arg-10 a*	FGSC[c]
FGSC8593	*dim-2*1 *arg-10 A*	FGSC[c]
KBNCR01A	*Sad-1*RIP64*; fl*p *A*	???
KBNCR02A	*Sad-1*RIP64*; fl*p *a*	???
KBNCR05A	*his-3*1-234-723*, Rsp*RIP93*; fl*p *A*	Sms-3 paper
KBNCR06A	*Rsp*RIP93*; fl*p *a*	Sms-3 paper
KYNCT02A	*his-3*1-234-723*; Asm-1*Δ(3430-9336)*::hph*+; *inl*89601 *a*	(Lee *et al.* 2004)
KYNCT13A	*his-3*+*::Asm-1*+[4615-6690]; *Asm-1*Δ(3430-9336)*::hph*+; *inl*89601 *a*	Transformation of KYNCT02A with pKYAM071
MMNCR01A	*fl*p*; Sms-3*RIP65 *A*	Sms-3 paper
MMNCR11A	*his-3*1-234-723*; fl*p*; Sms-3*RIP65 *a*	Sms-3 paper
MMNCR20A	*his-3*1-234-723*; Sms-3*RIP19*; inl*89601 *a*	Sms-3 paper
RANCR05A	*his-3*1-234-723*; inl*89601*A*	RANC collection
RANCR06A	*his-3*1-234-723*; inl*89601 *a*	RANC collection
RANCR49A	*fl*p *A*	RANC Collection
RANCR50A	*fl*p *a*	RANC collection
RLM 25-24	*rid-1*RIP, *his-3*+BM::*ccg-1*(p)::*rfp*+::*sad-2*+; *inv*, *Sad-2*RIP32 *A*	(Shiu *et al.* 2006)
RLM 25-25	*rid-1*RIP, *his-3*+BM::*ccg-1*(p)::*rfp*+::*sad-2*+; *inv*, *Sad-2*RIP32 *a*	(Shiu *et al.* 2006)
RLM 30-12	*Sad-2*RIP32 *A*	(Shiu *et al.* 2006)
RLM 42-32	*pan-2 a*	Robert L. Metzenberg
RPNCR23A	*his-3::hph*+*::tk*+*::lpl*Δ(5192-6064)*; inl*89601*; Sms-2*RIP88 *A*	DLNCR62A X DLNCR88A
RPNCR23C	*his-3::hph*+*::tk*+*::lpl*Δ(5192-6064)*; inl*89601*; Sms-2*RIP88 *A*	DLNCT62C X RPNCR40A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR38A	*mep*10 *his-3*1-234-723; *inl*89601; *pan-2*Δ3'::*hph*$^+$::*tk*$^+$ *A*	To be described elsewhere
RPNCR4A	*his-3*1-234-723; *inl*89601; *qde-2*RIP40; *Sms-2*RIP88 *a*	RPNCT28A X RANCR06A
RPNCR43A	*rsp*RIP94; *fl*p; *inl*89601 *a*	RANCR50A X RPNCR83A
RPNCR44A	*rsp*RIP94; *fl*p *A*	Sibling RPNCR43A
RPNCR45A	*fl*p; *inl*89601; *dim-2*^{1} *a*	Sibling RPNCR43A
RPNCR46A	*fl*p; *inl*89601; *dim-2*^{1} *A*	RPNCR45A X RPNCR83A
RPNCR62A	*inl*89601; *pan-2*Δ*3'::hph*$^+$*::tk*$^+$ *a*	To be described elsewhere
RPNCR63A	*inl*89601; *pan-2*Δ*3'::hph*$^+$*::tk*$^+$ *A*	Sibling of RPNCR62A
RPNCR66A&B	*A*	FGSC8592 X DLNCR93A
RPNCR67A	*his-3*1-234-723 *a*	RPNCR75A X DLNCR93A
RPNCR68A-D	*inl*89601 *a*	RPNCR76A X DLNCR93A
RPNCR69A	*his-3*1-234-723 *A*	Sibling RPNCR67A
RPNCR7A&B	*his-3*1-234-723; *inl*89601 *A*	Sibling RPNCR68A
RPNCR71A&B	*Rsp*RIP93 *a*	Sibling RPNCR66A
RPNCR72A	*his-3*1-234-723, *Rsp*RIP93 *a*	Sibling RPNCR67A
RPNCR73A	*Rsp*RIP93; *inl*89601 *a*	Sibling RPNCR66A
RPNCR73B	*Rsp*RIP93; *inl*89601 *a*	Sibling RPNCR68A
RPNCR74A	*his-3*1-234-723, *Rsp*RIP93; *inl*89601 *A*	Sibling RPNCR67A
RPNCR75A	*his-3*1-234-723; *dim-2*^{1} *a*	Sibling RPNCR66A
RPNCR76A	*inl*89601; *dim-2*^{1} *a*	Sibling RPNCR66A
RPNCR76B&C	*inl*89601; *dim-2*^{1} *a*	RPNCR76A X RPNCR82B
RPNCR76D	*inl*89601; *dim-2*^{1} *a*	Sibling RPNCR68A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR77A	*his-3*1-234-723*; dim-2*^{1} *A*	Sibling RPNCR67A
RPNCR78A	*his-3*1-234-723*; inl*89601*; dim-2*^{1} *A*	Sibling RPNCR76B
RPNCR79A	*Rsp*RIP93*; inl*89601*; dim-2*^{1} *a*	Sibling RPNCR76B
RPNCR79B	*Rsp*RIP93*; inl*89601*; dim-2*^{1} *a*	Sibling RPNCR68A
RPNCR81A	*his-3*1-234-723*, Rsp*RIP93*; dim-2*^{1} *A*	Sibling RPNCR67A
RPNCR82A&B	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*^{1} *A*	Sibling RPNCR66A
RPNCR82C&D	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*^{1} *A*	Sibling RPNCR76B
RPNCR82E&F	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*^{1} *A*	Sibling RPNCR68A
RPNCR83A	*rsp*RIP94*; inl*89601*; dim-2*^{1} *A*	DLNCR94A X FGSC8593
RPNCR84A&B	*Rsp; inl*89601*; dim-2*^{1}*, arg-10 A*	FGSC5892 X ACNC1
RPNCR85A	*Sad-1*RIP64*; dim-2*^{1}*, arg-10 A*	FGSC5892 X DLNCR123A
RPNCR86A	*Sad-1*RIP64*, his-3*1-234-723*; dim-2*^{1}*, arg-10 A*	Sibling RPNCR85A
RPNCR87A	*Sad-1*$^{\Delta}$*::hph*$^{+}$*; dim-2*^{1}*, arg-10 A*	FGSC8592 X FGSC8740
RPNCR89A	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*RIP89 *A*	RPNCR88A X DLNCR93A
RPNCR90A	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*RIP90 *A*	Sibling RPNCR90A
RPNCR91A	*his-3*1-234-723*, Rsp*RIP93*; inl*89601*; dim-2*RIP90 *a*	RPNCR90A X RANCR6A
RPNCR92A	*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Rsp*[1123-6375] *A*	DLNCT115A X RPNCR75A
RPNCR93A	*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Rsp*[1123-6375]*; inl*89601 *A*	Sibling RPNCR92A
RPNCR94A&B	*his-3*$^{+}$*::lpl*$^{\Delta}$(5192-6046)*::Rsp*[1123-6375]*; dim-2*^{1} *A*	Sibling RPNCR92A
RPNCR95A	*his-3*1-234-723*, Rsp*RIP95*; dim-2*^{1} *a*	RPNCR94A X RPNCR75A
RPNCR96A	*his-3*1-234-723*, Rsp*RIP96*; dim-2*^{1} *a*	Sibling RPNCR95A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR97A	*his-3*1-234-723, *Rsp*RIP97; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR98A	*his-3*1-234-723, *Rsp*RIP98; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR99A	*his-3*1-234-723, *Rsp*RIP99; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR100A	*his-3*1-234-723, *Rsp*RIP100; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR101A	*his-3*1-234-723, *Rsp*RIP101; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR102A	*his-3*1-234-723, *Rsp*RIP102; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR103A	*his-3*1-234-723, *Rsp*RIP103; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR104A	*his-3*1-234-723, *Rsp*RIP104; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR105A	*his-3*1-234-723, *Rsp*RIP105; *dim-2*1 *a*	Sibling RPNCR95A
RPNCR107A	*his-3*1-234-723, *Rsp*RIP95 *a*	RPNCR95A X ACNC9
RPNCR108A	*his-3*1-234-723, *Rsp*RIP96; *inl*89601 *A*	RPNCR96A X ACNC9
RPNCR109A	*his-3*1-234-723, *Rsp*RIP97 *A*	RPNCR97A X ACNC9
RPNCR110A	*his-3*1-234-723, *Rsp*RIP98 *A*	RPNCR98A X ACNC9
RPNCR111A	*his-3*1-234-723, *Rsp*RIP99 *a*	RPNCR99A X ACNC9
RPNCR112A	*his-3*1-234-723, *Rsp*RIP100; *inl*89601 *a*	RPNCR100A X ACNC9
RPNCR113A	*his-3*1-234-723, *Rsp*RIP101 *A*	RPNCR101A X ACNC9
RPNCR114A	*his-3*1-234-723, *Rsp*RIP102 *A*	RPNCR102A X ACNC9
RPNCR115A	*his-3*1-234-723, *Rsp*RIP103 *A*	RPNCR103A X ACNC9
RPNCR116A	*his-3*1-234-723, *Rsp*RIP104 *A*	RPNCR104A X ACNC9
RPNCR117A	*his-3*1-234-723, *Rsp*RIP105 *a*	RPNCR105A X ACNC9
RPNCR119A	*his-3*1-234-723, *Rsp*RIP97 *a*	Sibling RPNCR109A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR120A	*his-3*1-234-723, *Rsp*RIP104 *a*	Sibling RPNCR116A
RPNCR123A	*his-3::hph*$^{+}$*::tk*$^{+}$*::lpl*$^{\Delta}$(5192-6064); *inl*89601; *qde-2*RIP40 *A*	DLNCR83B X RPNCR40A
RPNCR142A	*Sms-4*UV; *pan-2*$^{\Delta 3'}$*::hph*$^{+}$*::tk*$^{+}$ *a*	S1 A X RPNCR62A
RPNCR143A	*Sms-4*UV; *pan-2*$^{\Delta}$3'*::hph*$^{+}$*::tk*$^{+}$ *A*	Sibling of RPNCR142A
RPNCR154A	*his-3*::*hph*$^{+}$*::tk*$^{+}$*::lpl*$^{\Delta}$(5192-6046), *Rsp*RIP93; *inl*89601 *a*	RPNCR82A X DLNCR83B
RPNCR158A	*his-3*1-234-723; *cyh-2*R, *Sms-4*UV, *inl*89601 *a*	BKNCR005 X RPNCR141A
RPNCR166A	*his-3*1-234-723; *mei-1* A	RPNCR115A X FGSC 2920
RPNCR167A	*his-3*1-234-723, *Rsp*RIP103; *mei-1* A	Sibling RPNCR166A
RPNCR168A	*Mei-2*ALS181 *A*	RPNCR143A X FGSC 5888
RPNCR169A	*his-3*1-234-723, *Rsp*RIP103; *Mei-2*ALS181 A	Sibling RPNCR168A
RPNCR173A	*his-3*1-234-723, *Rsp*RIP103; *inl? a*	Sibling of RPNCR174A
RPNCR174A	*his-3*1-234-723, *msh4*RIP174 *kinky A*	1) RPNCR38A was transformed with pRATT72 2) A transformant (*pan-2*$^{+}$) X RPNCR62A 3) Selected progeny (*pan-2*$^{+}$) X RPNCR103A
RPNCR178A	*his-3?; rca-1*$^{\Delta}$*::hph*$^{+}$ *a*	DE41 a X RPNCR115A
RPNCR179A	*his-3*1-234-723, *msh4*RIP174, *Rsp*RIP103 *A*	RPNCR174A X RPNCR115A
RPNCR181A	*his-3*1-234-723, *msh4*RIP174; *inl*89601 *a*	Sibling RPNCR179A
RPNCR182A	*Sad-1*$^{\Delta}$::*hph*$^{+}$, *his-3*1-234-723, *msh4*RIP174 *kinky A*	RPNCR174A X FGSC8740
RPNCR182B	*Sad-1*$^{\Delta}$::*hph*$^{+}$, *his-3*1-234-723, *msh4*RIP174 *A*	RPNCR182A X RPNCR66A
RPNCR183A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *mei-1 A*	FGSC 8740 X FGSC 2920
RPNCR184A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *Mei-2*ALS181 *A*	FGSC 8740 X FGSC 5888
RPNCR200A	*his-3*1-234-723; *NCU01309*$^{\Delta}$*::hph*$^{+}$*::npt, inl*89601 *a*	1) RANCR05A was transformed with pRATT78 2) A transformant (*hph*$^{+}$) X RPNCR72A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR201A	*his-3*1-234-723, *Rsp*RIP93; *NCU01309*Δ*::hph*+*::npt, inl*89601 *A*	Sibling of RPNCR200A
RPNCR202A	*his-3*1-234-723; *Sms-4*Δ*::hph*+*::npt, inl*89601 *A*	1) RANCR05A was transformed with pRATT79 2) A transformant (*hph*+) X RPNCR72A
RPNCR203A	*his-3*1-234-723; *Sms-4*Δ*::hph*+*::npt, inl*89601*a*	Sibling of RPNCR202A
RPNCR204A	*his-3*1-234-723, *Rsp*RIP93; *Sms-4*Δ*::hph*+*::npt, inl*89601 *A*	Sibling of RPNCR202A
RPNCR206A	*his-3*1-234-723; *NCU01311*Δ*::hph*+*::npt, inl*89601 *a*	1) RANCR05A was transformed with pRATT80 2) A transformant (*hph*+) X RPNCR72A
RPNCR207A	*his-3*1-234-723, *Rsp*RIP93; *NCU01311*Δ*::hph*+*::npt, inl*89601 *A*	Sibling of RPNCR206A
RPNCR208A	*his-3?, Rsp*RIP93; *rca-1*Δ*::hph*+ *A*	DLNCR93A X RPNCR178A
RPNCR210A	*his-3*1-234-723, *Rsp*RIP103; *Mei-2*ALS181 *a*	Sibling RPNCR169A
RPNCR211A	*Sms-4*UV, *Mei-2*ALS181 *A*	Sibling RPNCR168A
RPNCR218A	*Sms-4*Δ*::hph*+*::npt, inl*89601; *pan-2*Δ*3'::hph*+*::tk+ A*	RPNCR203A X RPNCR63A
RPNCR220A	*rid-1*RIP245, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064); *Sms-4*Δ*::hph*+*::npt, inl*89601*A*	RPNCR203A X DLNCR245A
RPNCR222A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064); *inl*89601; *dim-2*1 *a*	RPNCR78A X DLNCR246A
RPNCR223A	*mep(10)?, his-3*1-234-723, *Rsp*RIP93; *intergenic[NCU01311-12]-1*RIP223, *inl*89601; *pan-2 A*	1) RPNCR38A was transformed with pRATT81 2) A transformant (*pan-2*+) X RPNCR72A 3) A progeny (pan-2+::insert; inl) X RLM 42-32
RPNCR224A	*mep(10)?; intergenic[NCU01311-12]-1*RIP224, *inl*89601; *pan-2 a*	Sibling of RPNCR223A
RPNCR225A	*mep(10)?, his-3*1-234-723, *Rsp*RIP93; *intergenic[NCU01311-12]-2*RIP225, *inl*89601; *pan-2 A*	1) RPNCR38A was transformed with pRATT83 2) A transformant (*pan-2*+) X RPNCR72A 3) A progeny (pan-2+::insert; inl) X RLM 42-32
RPNCR226A	*mep(10)?, intergenic[NCU01311-12]-2*RIP226, *inl*89601; *pan-2 a*	Sibling of RPNCR225A
RPNCR231A	*rid-1*RIP245, *his-3::hph*+*::tk+::lpl*Δ(5192-6064); *Sms-4*UV *A*	S1 a X DLNCR245A
RPNCR232A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064); *Sms-4*UV *a*	S1 A X DLNCR246A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR237A	*Sad-1*Δ*::hph*+, *rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064) *a*	FGSC8740 X DLNCR246A
RPNCR238A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064); *inl*89601; *Sms-2*RIP88 *a*	RPNCR23C X DLNCR246A
RPNCR240A	*his-3*1-234-723, *Rsp*Δ[2516-6316]*::hph*+; *Sms-4*Δ*::hph*+*::npt, inl*89601 *A*	RPNCR203A X DLNCT212A
RPNCR241A	*Rsp*Δ(2516-6316)*::hph*+; *Sms-4*UV *A*	S1 a X DLNCT212A
RPNCR242A	*his-3*1-234-723, *Rsp*RIP103; *Sms-4*Δ*::hph*+*::npt, inl*89601 *a*	RPNCR103A X RPNCR202A
RPNCR243A	*his-3*1-234-723, *Rsp*RIP103; *Sms-4*Δ*::hph*+*::npt, inl*89601; *dim-2*1 *a*	Sibling of RPNCR242A
RPNCR244A	*Rsp*RIP93; *Sms-4*Δ*::hph+::npt, inl*89601 *a*	RPNCR79A X RPNCR202A
RPNCR248A	*rid-1*RIP245, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064), *Rsp*RIP93; *inl*89601 *A*	RPNCR154A X DLNCR245A
RPNCR249A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064), *Rsp*RIP93; *inl*89601 *a*	RPNCR74A X DLNCR246A
RPNCR250A	*Rsp*RIP93; *Sms-4*UV, *inl*89601*A*	RPNCR160A X RPNCR72A
RPNCR251A	*rid-1*RIP246, *his-3*+*::sms-4*+[*Hind*III]; *Sms-4*UV *a*	RPNCR232A X RPNCR239A
RPNCR253B	*rid-1*RIP246, *his-3*+*::Asm-1*+[9336-3430]; *Sms-4*Δ*::hph*+*::npt, inl*89601 *a*	RPNCR220A X DLNCT291A
RPNCR266A	*his-3::hph*+*::tk*+*::lpl*Δ(5192-6064); *Sms-4*Δ*::hph*+*::npt, inl*89601 *A*	RPNCR203A X DLNCR62C
RPNCR267A	*rid-1*RIP245, *his-3*+*::sms-4*+[*Hind*III], *Rsp*RIP93; *Sms-4*UV *A*	RPNCR249AX RPNCT239A
RPNCR269A	*rid-1*RIP246, *his-3::hph*+*::tk*+*::lpl*Δ(5192-6064), *rsp*RIP94; *inl*89601 *a*	RPNCR44A X RPNCR222A
RPNCR272A	*his-3*1-234-723, *Rsp*Δ(2516-6316)*::hph*+; *mei-1*; *inl*89601 *a*	RPNCR166A X DLNCR213A
RPNCR274A	*his-3*1-234-723, *msh4*RIP174, *Rsp*Δ(2516-6316)*::hph*+; *inl*89601 *a*	RPNCR241A X RPNCR181A
RPNCR275A	*Rsp*RIP93; *Sms-4*UV; *pan-2*Δ3'*::hph*+*::tk*+ *a*	RPNCR143A X RPNCR73A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR279A	*rid-1*RIP245*, his-3*$^{+}$*::Asm-1*$^{+}$[9336-3430]*; Sms-4*UV *A*	RPNCR232A X DLNCT290A
RPNCR280A	*rid-1*RIP246*, his-3*$^{+}$*::sms-4*$^{+}$[*Hind*III]*; Sms-4*$^{\Delta}$*::hph*$^{+}$*::npt, inl*89601 *a*	RPNCR251A X RPNCR220A
RPNCR283A	*rid-1*RIP245*, his-3::hph*$^{+}$*::tk*$^{+}$*::lpl*$^{\Delta}$(5192-6064)*, Rsp*RIP93*; Sms-4*UV *A*	RPNCR251A X RPNCR248A
RPNCR286B	*rid-1*RIP246*, his-3*$^{+}$*::sms-4*W97A[*Bam*HI-*Hind*III]*; Sms-4*UV *a*	1) RANCR05A was transformed with pRATT107 2) A transformant (*his*$^{+}$) X RPNCR232A
RPNCR287B	*rid-1*RIP245*, his-3*$^{+}$*::sms-4*W97A[*Bam*HI-*Hind*III]*, Rsp*RIP93*; Sms-4*UV *A*	1) RANCR05A was transformed with pRATT107 2) A transformant (*his*$^{+}$) X RPNCR249A
RPNCR292A	*ski8*RIP292; *inl*89601; *pan-2*$^{\Delta}$3'::*hph*$^{+}$::*tk*$^{+}$ *a*	1) RPNCR38A was transformed with pRATT105 2) A transformant (*pan-2*$^{+}$) X RPNCR275A
RPNCR293A	*his-3*1-234-723, *Rsp*RIP93; *ski8*RIP293; *inl*89601; *pan-2*$^{\Delta}$::*hph*$^{+}$::*tk*$^{+}$ *A*	Sibling RPNCR292A
RPNCR294A	*spo11*RIP294, *inl*89601; *pan-2*$^{\Delta}$3'::*hph*$^{+}$::*tk*$^{+}$ *a*	1) RPNCR38A was transformed with pRATT106 2) A transformant (*pan-2*$^{+}$) X RPNCR275A
RPNCR295A	*his-3*1-234-723, *Rsp*RIP93; *spo11*RIP295, *inl*89601; *pan-2*$^{\Delta}$3'::*hph*$^{+}$::*tk*$^{+}$ *A*	Sibling RPNCR294A
RPNCR303A	*rid-1*RIP246*, his-3::hph*$^{+}$*::tk*$^{+}$*::lpl*$^{\Delta}$(5192-6064)*; inl*89601*; qde-1*$^{\Delta}$*::hph*$^{+}$ *a*	DLNCR246A X DLNCT117A
RPNCR304A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *spo11*RIP294, *inl*89601 *A*	RPNCR294A X FGSC 8741
RPNCR305A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *spo11*RIP294, *inl*89601 *a*	Sibling RPNCR304A
RPNCR306A	*mei-3*C325*; *spo11*RIP294, *inl*89601 *A*	FGSC 6187 X RPNCR294A
RPNCR307B	*mei-3*C325*, *Rsp*RIP93; *spo11*RIP295, *inl*89601 *a*	RPNCR295A X FGSC 6188
RPNCR316A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *ski8*RIP292; *inl*89601 *A*	RPNCR292A X FGSC 8741
RPNCR317A	*Sad-1*$^{\Delta}$::*hph*$^{+}$; *ski8*RIP292 *a*	Sibling RPNCR316A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR318A	*mei-3*C325*; *ski8*RIP293 *A*	FGSC 6187 X RPNCR293A
RPNCR319A	*mei-3*C325*, *Rsp*RIP93; *ski8*RIP292; *inl*89601 *a*	RPNCR292A X FGSC 6188
RPNCR320A	*Sms-4*Δ*::hph*RIP320*::npt*, *inl*89601; *qde-1*Δ*::hph*+ *A*	RPNCR303A X RPNCR218A
RPNCR321A	*flp; Sms-4*Δ*::hph*RIP?*::npt, inl*89601 *A*	RPNCR320A X KBNCR06A
RPNCR326A	*inl*89601 *A*	RPNCR292A X RPNCR44A
RPNCR327A	*rsp*RIP94; *spo11*RIP294, *inl*89601 *a*	RPNCR294A X RPNCR44A
RPNCR328A	*spo11*RIP294, *inl*89601 *A*	Sibling RPNCR327A
RPNCR329A	*spo11*RIP294, *inl*89601 *a*	Sibling RPNCR327A
RPNCR330A	*ski8*RIP292; *inl*89601 *a*	Sibling RPNCR326A
RPNCR331A	*A*	1) RPNCR320A X KBNCR06A 2) a progeny X RANCR50A
RPNCR333A	*Sms-4*Δ*::hph*RIP333*::npt A*	Sibling of RPNCR331A
RPNCR334A	*rsp*RIP94; *inl*89601 *A*	Sibling RPNCR326A
RPNCR336A	*rid-1*RIP246*, his-3*+*::sms-4*W97*fs[*Bam*HI-*Hind*III]*; Sms-4*UV *a*	1) RANCR05A was transformed with pRATT107*) A transformant (*his*+) X RPNCR232A
RPNCR337A	*rsp*RIP94; *ski8*RIP293; *inl*89601 *a*	RPNCR293A X RPNCR43A
RPNCR338A	*rid-1*RIP245*, his-3*+*::sms-4*W97*fs[*Bam*HI-*Hind*III], *Rsp*RIP93*; Sms-4*UV *A*	1) RANCR05A was transformed with pRATT107* 2) A transformant (*his*+) X RPNCR249A
RPNCR369A	*rid-1*RIP246; *inl*89601 *a*	S1A X DLNCR246A
RPNCR370A	*rid-1*RIP246*; Sms-4*UV *a*	Sibling RPNCR369A
RPNCR375A	*mus-21*FK120; *inl*89601 *a*	RPNCR62A X FGSC 6418
RPNCR376A	*inl*89601, *mus-11*FK117 *a*	RPNCR62A X FGSC 6409
RPNCR383A	*mus-21*FK120; *spo11*RIP294, *inl*89601 *A*	RPNCR329A X FGSC 6418

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR384A	*mus-21*FK120; *spo11*RIP294, *inl*89601 *a*	Sibling RPNCR383A
RPNCR385A	*spo11*RIP294, *inl*89601, *mus-11*FK117 *A*	RPNCR329A X FGSC 6409
RPNCR386A	*spo11*RIP294, *inl*89601, *mus-11*FK117 *a*	Sibling RPNCR385A
RPNCR387A	*mus-9*FK104; *spo11*RIP294, *inl*89601 *A*	RPNCR329A X FGSC 5146
RPNCR388A	*mus-9*FK104; *spo11*RIP294, *inl*89601 *a*	Sibling RPNCR387A
RPNCR390A	*rid-1*RIP246, *mei-3*C325*; *inl*89601 *a*	DLNCR246A X FGSC 6187
RPNCR392A	*rid-1*RIP246, *mus-9*FK104; *inl*89601 *a*	DLNCR246A X FGSC 5146
RPNCR396A	*rid-1*RIP245*; Sms-4*Δ*::hph*RIP396*::npt, inl*89601*; pan-2*Δ*3'::hph*+*::tk*+ *A*	RPNCR220A X RPNCR142A
RPNCR397A	*rid-1*RIP245*; Sms-4*UV *A*	Sibling RPNCR396A
RPNCR410A	*rid-1*RIP246*, his-3::hph*+*::tk*+*::lpl*Δ(5192-6046)*; Sad-2*UV *a*	S10 A X DLNCR246A
RPNCR416A	*rid-1*RIP245; *Sms-4*UV; *hH1*+::*sgfp*+::*bla*+::*hph*+ *A*	1) DLNCR316A was transformed with pRATT120 2) A transformant (*hph*+) X RPNCR397A
RPNCR417A	*rid-1*RIP246; *inl*89601; *hH1*+::*sgfp*+::*bla*+::*hph*+ *a*	1) DLNCR315A was transformed with pRATT120 2) A transformant (*hph*+) X RPNCR369A
RPNCR430A	*rid-1*RIP246, *mei-3*C325*; *inl*89601; *hH1*+::*sgfp*+::*bla*+::*hph*+ a	RPNCR390A X RPNCR416A
RPNCR472A	*rid-1*RIP245*; inl*89601*; hH1*+*::sgfp*+*::bla::hph*+ *A*	RPNCR417A X RPNCT355A
RPNCR475A	*rid-1*RIP245*; inl*89601*; Sms-2*RIP88*, hH1*+*::sgfp*+*::bla::hph*+ *A*	RPNCR238A X RPNCR416A
RPNCR477A	*his-3*1-234-723; *inl*89601; *hH1*+::*sgfp*fs::*bla*+::*hph*+ a	1) DLNCR316A was transformed with pRATT120* 2) A transformant (*hph*+) X RPNCR397A
RPNCR482A	*mei-3*C325*; *inl*89601; *hH1*+::*sgfp*fs::*bla*+::*hph*+ A	FGSC 6187 X RPNCR477A
RPNCR487A	*Sad-1*Δ*::hph*+*, rid-1*RIP245*; hH1*+*::sgfp*+*::bla::hph*+ *A*	RPNCR237A X RPNCR416A
RPNCR499D	*sms-4*+*::sgfp***::bla::hph*+*, inl*89601 *A*	1) DLNCR316A was transformed with pRATT122*

Table A1. Continued

Name[a]	Genotype[b]	Origin
		2) A transformant (*hph*$^+$) X RPNCR331A
RPNCR500A	*rid-1*RIP245*; sms-4*$^+$*::sgfp***::bla::hph*$^+$*, inl*89601 *A*	1) DLNCR316A was transformed with pRATT122* 2) A transformant (*hph*$^+$) X RPNCR397A
RPNCR505A	*rid-1*RIP246*; sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *a*	1) DLNCR315A was transformed with pRATT122 2) A transformant (*hph*$^+$) X RPNCR369A
RPNCR506A	*sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *A*	1) DLNCR315A was transformed with pRATT122 2) A transformant (*hph*$^+$) X RPNCR68A
RPNCR507A	*his-3*1-234-723*; sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *A*	Sibling of RPNCR506A
RPNCR508A	*sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *a*	Sibling of RPNCR506A
RPNCR509A	*his-3*1-234-723*; sms-4*$^+$*::sgfp***::bla::hph*$^+$*, inl*89601 *a*	Sibling of RPNCR499D
RPNCR521A	*Sad-1*Δ*::hph*$^+$*; sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *a*	FGSC 8741 X RPNCR507A
RPNCR522A	*sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601*, Sad-2*UV *a*	S10 A X RPNCR508A
RPNCR523A	*rid-1*RIP246*, his-3*$^+$*::ccg-1(p)::rfp*RIP*::sms-2; sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601*; Sms-2*RIP88 *a*	DLNCT389A X RPNCR507A
RPNCR524A	*rid-1*RIP246*, his-3*$^+$*::ccg-1(p)::rfp*RIP*::sms-3; sms-4*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, inl*89601 *a*	DLNCT391A X RPNCR507A
RPNCR531A	*Rsp*RIP93*; sms-4*$^+$*::sgfp***::bla::hph*$^+$*, inl*89601 *A*	RPNCR73A X RPNCR499D
RPNCR533A	*sms-4*$^+$*::sgfp***::bla::hph*$^+$*, inl*89601*, Sad-2*UV *A*	RPNCR410A X RPNCR499D
RPNCR534A	*Sms-3*RIP19*; sms-4*$^+$*::sgfp***::bla::hph*$^+$*, inl*89601 *A*	MMNCR20A X RPNCR499D
RPNCR548A	*sad-1*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, rid-1*RIP245*; inl*89601 *A*	1) DLNCR316A was transformed with pRATT123 2) A transformant (*hph*$^+$) X RPNCR397A
RPNCR549A	*sad-1*$^+$*::sgfp*$^+$*::bla::hph*$^+$*; inl*89601 *a*	Sibling RPNCR548A
RPNCR552A	*sad-1*$^+$*::sgfp*$^+$*::bla::hph*$^+$*, rid-1*RIP245*; Sms-4*UV *A*	Sibling RPNCR548A

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCR553A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, his-3*$^{1\text{-}234\text{-}723}$*; Sms-4*^{UV} *a*	Sibling RPNCR548A
RPNCR555A	*rid-1*^{RIP}*, his-3*$^{+}$BM::*ccg-1*(p)::*rfp*$^{+}$::*sad-2*$^{+}$*; sms-4*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, inl*^{89601} *A*	RPNCR505A X RLM 25-24
RPNCR556A	*rid-1*^{RIP}*, his-3*$^{+}$BM::*ccg-1*(p)::*rfp*$^{+}$::*sad-2*$^{+}$*; sms-4*$^{+}$*::sgfp***::bla::hph*$^{+}$*, inl*^{89601} *a*	RPNCR500A X RLM 25-25
RPNCR557A	*rid-1*^{RIP}*, his-3*$^{+}$BM::*ccg-1*(p)::*rfp*$^{+}$::*sad-2*$^{+}$*; Sms-4*^{UV} *a*	RPNCR397A X RLM 25-25
RPNCR560A	*rid-1*^{RIP246}*, his-3*$^{+}$*::ccg-1(p)::rfp*^{RIP}*::sms-3; Sms-3*^{RIP19}*; sms-4*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, inl*^{89601} *a*	RPNCR524A X RPNCR534A
RPNCR570A	*Rsp*^{RIP93}*; sms-4*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, inl*^{89601} *A*	RPNCR506A X KBNCR06A
RPNCR571A	*rid-1*^{RIP}*, his-3*$^{+}$BM::*ccg-1*(p)::*rfp*$^{+}$::*sad-2*$^{+}$*, Rsp*^{RIP93}*; inv, Sad-2*^{RIP32} *A*	RLM 25-24 X RPNCR249A
RPNCR572A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, rid-1*^{RIP245}*, Rsp*^{RIP93}*; inl*^{89601} *A*	RPNCR249A X RPNCR548A
RPNCR573A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*; Sad-2*^{RIP32} *A*	RLM 30-12 X RPNCR549A
RPNCR574A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*; Sad-2*^{RIP32} *a*	Sibling RPNCR573A
RPNCR575A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, mei-3*^{C325*}*; inl*^{89601} *A*	FGSC 6187 X RPNCR549A
RPNCR576A	*sad-1*$^{+}$*::sgfp*$^{+}$*::bla::hph*$^{+}$*, mei-3*^{C325*} *a*	FGSC 6188 X RPNCR548A
RPNCR586A	*Sad-1*$^{\Delta}$*::hph*$^{+}$*; sms-4*$^{+}$*::sgfp***::bla::hph*$^{+}$*, inl*^{89601} *A*	FGSC 8740 X RPNCR509A
RPNCR587A	*Sad-1*$^{\Delta}$*::hph*$^{+}$*; inl*^{89601}*; hH1*$^{+}$*::sgfp*^{fs}*::bla::hph*$^{+}$ *a*	RPNCR476A X FGSC 8741
RPNCR588A	*his-3*$^{1\text{-}234\text{-}723}$*; inl*^{89601}*; Sms-2*^{RIP88}*, hH1*$^{+}$*::sgfp*^{fs}*::bla::hph*$^{+}$ *a*	RPNCR476A X DLNCR88A
RPNCT028A	*his-3*$^{+}$*::sms-2(p)::qde-2*^{ORF}*::sms-2(t); inl*^{89601}*; Sms-2*^{RIP88} *A*	Transformation of RPNCR23A with pRATT39a1
RPNCT088A	*his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::dim-2*[1513-2469]*, Rsp; inl*^{89601} *a*	Transformation of DLNCR99A with pRATT47
RPNCT140A	*his-3*$^{+}$*::ccg-1*(p)::*al-1*^{hp}::*ccg-1*(t); *inl*^{89601} *A*	Transformation of DLNCR62C with pRATT67
RPNCT189A	*his-3*$^{+}$*::ccg-1*(p)::*al-1*^{hp}::*ccg-1*(t); *inl*^{89601}*; qde-1*$^{\Delta}$*::hph*$^{+}$ *A*	Transformation of DLNCT117A with pRATT67

Table A1. Continued

Name[a]	Genotype[b]	Origin
RPNCT239A	*rid-1*RIP245*, his-3*$^{+}$*::sms-4*$^{+}$[*Hind*III]*; Sms-4*^{UV} *A*	Transformation of RPNCR231A with pRATT100
RPNCT355A	*rid-1*RIP245*; Sms-4*$^{\Delta}$*::hph*RIP396*::npt, inl*89601*; pan-2*$^{+}$*::ccg-1(p)::sgfp*^{hp} *A*	Transformation of RPNCR396A with pRATT113
RPNCT414A	*ski8*RIP292; *inl*89601; *pan-2*$^{+}$::*ccg-1*(p)::*hH1*$^{+}$::*sgfp*$^{+}$ [→] *a*	RPNCR292A was transformed with pRATT116
RPNCT415A	*spo11*RIP294, *inl*89601; *pan-2*$^{+}$::*ccg-1*(p)::*hH1*$^{+}$:: *sgfp*$^{+}$ [→] *a*	RPNCR294A was transformed with pRATT116
RPNCT567A&B	*his-3*$^{+}$*::ccg-1*(p)::*al-1*^{hp}::*ccg-1*(t); *Sms-4*$^{\Delta}$*::hph*$^{+}$*::npt, inl*89601 *A*	Transformation of RPNCR266A with pRATT67
S1 A	*Sms-4*^{UV} *A*	To be described elsewhere
S1 a	*Sms-4*^{UV} *a*	To be described elsewhere
S1 A	*Sms-4*^{UV} *A*	Product of mutagenesis
S1 a	*Sms-4*^{UV} *a*	Product of mutagenesis
S10 A	*Sad-2*^{UV} *A*	Product of mutagenesis
TGNCT01A&B	*his-3*$^{+}$*::ccg-1*(p)::*al-1*^{hp}::*ccg-1*(t); *inl*89601*; qde-2*RIP40 *A*	Transformation of RPNCR123A with pRATT67

[a]ACNC, DLNC, KBNC, MMNC, RANC, RPNC and TGNC indicate strains constructed or provided for this study by Alejandro Correa, Dong W. Lee, Kevin D. Baker, Malcolm T. McLaughlin, Rodolfo Aramayo, Robert J. Pratt, and Todd Gruninger, respectively.

[b]Detailed descriptions of all loci can be found at "The Neuropsora e-Compendium" linked at <www.fgsc.net> or are given in text. Locus names are given with allele designations suffixed as superscripts. Alleles designated with a * indicate a nonsense mutation, fs designates a frameshift mutation and hp designates a hairpin. Double colons signify a fusion between the genes, although not necessarily a translational or transcriptional fusion, see text for details. The symbols (p) and (t) following a gene indicate the promoters and terminators for those genes. In the interest of space *Rsp* alleles were abbreviated in the text, however the correct nomenclature is used in this table; *Rsp* = *Rsp*$^{\Delta(1)}$, *Rsp*$^{\Delta(2516\text{-}6316)}$*::hph*$^{+}$= *Rsp*$^{\Delta(2)}$, *his-3*$^{+}$*::lpl*$^{\Delta(5192\text{-}6046)}$*::Rsp*[1123-6375] = *Rsp*^{ect}.

[c]Construction of the different plasmids is described in Materials and Methods.

[d]FGSC, indicates strains acquired from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City (McCluskey 2003).

APPENDIX B

Table B1. Oligonucleotides used in this study

Name	Sequence
His3D	GTCGTCCACAGCCGCCCAACTC
His3U	GTCTGTGCAATCCCCCAATCCA
ODL159	GTCGAATTCTCAAGCCCATCAATCATCAATCGGTCAA
ODL160	AAGGGATCCTGGAGGCGCGATTTAGGGGTGCTGTTAC
ORP014	CGGATCCGACGTGTAATGCTGC
ORP027	ATTTAAATCTAAGGAGGATATTC
ORP031	AGATCTGAAGCGGCGCACG
ORP066	GGTCTAGATGGTCGCTTTGGCT
ORP067	GGTCTAGACATTTTGCAGGTAC
ORP072	TGTCTAGACTTTCGCTCAGCGAGAAGGAGA
ORP073	CCTCTAGATTAGATATAGTACATGGAGTTC
ORP083	AGCTACACGACTTCAACTTCT
ORP084	TCATCCGTGTCCGTTGGTTTT
ORP111	GGTCTGCAGCTTGTACAGCTCGTC
ORP113	GTGGCGGCCGCAGATGTGCCTCACCTCACCGT
ORP114	CGTGCGGCCGCATTGGAGCCATCTTGAGTCCA
ORP115	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCAACGAACTGAATCTCAAACAAC GTCCT
ORP116	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGTGCAGGTGAGAACGGTAGGG CGGTGA
ORP128	GAGGATCCAGTTCCAACCGGGCGC
ORP129	GGTCTAGACATTTTGGTTGATGTG
ORP130	CCTCTAGAGCGACTTTACCAACAG
ORP131	CCGAATTCTGGACGCCATCTTGTA
ORP134	CCACTAGTGCATTGCCGTCATCA
ORP135	CCACTAGTCCCACCCGTCCTTGT
ORP148	GGGCTGCAGTACGCGGGACTCTAC
ORP149	GCGGATCCGGCTCCGTATGAGGT
ORP150	GCGGATCCATGCATGGTCACCTACCAGCA
ORP151	GCGGATCCTGCAGTGGAGCAGTTTGCT
ORP152	GCGGATCCTGCAGATTAGACGACGGCAC
ORP153	GCGGATCCATGCATATATCGGCTGCTGAC
ORP154	GCGGATCCAGCCGTTGAACCTTT
ORP155	CCCCTGCAGCCTGAGTCGTGTCCA
ORP156	GCAGCCGGGAGAAGGGTCGGGCGATCA
ORP157	GCGGGCCGCAGCACCGACGACTTTCCT
ORP170	CACCATGGATTTAGATATTGAGATGGA
ORP171	CTAATAGAACATATCCTCAGCTCT
ORP177	GCCTCCACCCTGTCGAGTTG
ORP178	CATCGATGACACCAATGCAA
ORP179	CCTTCTCAACCCACGGCT
ORP180	CGGAGCAATCTTGCGTCA
ORP183	CTCTACATTCGCCTCGTG
ORP184	CCAGATAGGGCTGGTTGA
ORP186	TTACTCCACCCTGTCGAGTTG
ORP202	CGAGCCGCCCACAACCTA
ORP203	CCTAAGCTTGCGCCTCGGTCTCTTCTC
ORP204	CTTGGATCCAAGAAAGTCAAGCA

Table B1. Continued

Name	Sequence
ORP205	CTCACTAGTTTCGTCATTCATAGC
ORP206	AGGAAGCTTGAGGGATAAGGAGT
ORP207	CTCGGATCCTTAACCTCGCTCAG
ORP208	ATCCGGCGGGGCCAATGGTGT
ORP209	GCTGTCGCCATCCCCACTGCA
ORP212	GCCGGATCCAGACTGAGGTCGTA
ORP213	GCCTCTAGAATAGAACATATCCTCAG
ORP214	GGCAAGCTTACGACGGCACCTCGG
ORP215	CCGCTGCAGCAACCTTAGCCCGGT
ORP216	GCCTCTAGAAAGCGCCGCCATCTG
ORP217	GGCAAGCTTGGCTCGTTGAGACCA
ORP218	GGCCTGCAGCATCGGGGCAGAGTA
ORP225	ATGGGCTGATGGTCGTCT
ORP226	GACACCGCCTAGTTGGCT
OTL001	GTGACTAGTGTGAGCAAGGGCGAG

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